BACTERIAL DIVERSITY IN THE BENTHIC ENVIRONMENT ALONG KERALA COAST AND THEIR POTENTIAL FOR EXTRACELLULAR ENZYME PRODUCTION

Thesis Submitted To

Cochin University Of Science And Technology

In the partial fulfiment of the requirement s for

The award of the degree of

Doctor of philosophy

in

Marine biology

Under the Faculty of Marine Sciences

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CERTIFICATE

This is to certify that the thesis entitled **"Bacterial Diversity in the Benthic environment along Kerala Coast and their potential for extracellular enzyme production",** is an authentic record of research work carried out by **Mr. Abhilash K. R**. (Reg. No. 2896) under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Marine Biology. All the relevant corrections and modifications suggested by audience and recommended by the Doctoral Committee have been incorporated in this thesis and no part thereof has been presented before for the award of any degree, diploma or associateship in any University.

August 2015
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Prof. (Dr.) A. V. Saramma
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DECLARATION

I hereby declare that the thesis entitled **"Bacterial Diversity in the Benthic environment along Kerala Coast and their potential for extracellular enzyme production"** is a genuine record of research work done by me under the supervision of **Dr. A.V. Saramma,** Professor, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi- 682016, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Marine Biology and that no part of this work, has previously formed the basis for the award of any degree, diploma associateship, fellowship or any other similar title of any University or Institution.

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Acknowledgements

Perseverance and Faith has finally led me to the completion of my doctoral thesis. I am deeply indebted to my guide Dr. A.V. Saramma, Professor, Department of Marine Biology, Microbiology and Biochemistry, CUSAT, for her whole-hearted support and impeccable guidance through the course of this work. Her valuable suggestions, stable and earnest encouragement, fruitful discussions and critical evaluation has been my constant motivation through the course of this work.

I gratefully acknowledge Dr. Rosamma Philip, Head, Department of Marine Biology, Microbiology and Biochemistry, CUSAT, for her encouragement and for providing facilities for undertaking the research work at the Department. My sincere thanks are also due to Dr. A.N. Balchand, Dean, Faculty of Marine Sciences and Dr. Sajan K., Director, Faculty of Marine Sciences for providing all the facilities for research. I am also thankful to the former Deans, Dr. Mohan Kumar and Dr. Ram Mohan and Dr. K. T. Damodaran for their support and goodwill.

I express my heartfelt gratitude to Prof. (Dr.) Babu Philip (Retd.), for his valuable suggestions and support as subject expert and Doctoral Committee Member. I express my profound sense of gratitude to all the teachers in the Department of Marine Biology, Dr. Aneykutty Joseph, Dr. A.A. Mohamed Hatha, Dr. S. Bijoy Nandan, Dr. N.R. Menon (Retd.), Dr. R. Damodaran (Retd.), Dr. K.J. Joseph (Retd.) Dr. C.K. Radhakrishnan (Retd.), Dr. V.J. Kuttiyamma (Retd.) and Dr. K.Y. Muhammad Salih (Late) for their valuable advice, suggestions and support. I also thank the non-teaching staff of the department for their help and good wishes. The help rendered by Mr. Stephen V.K., Mr. Balan V.K., Mr. Abdul Nazar M., Mr. Sanjiv T.K. and Mr. Santhosh S. is gratefully acknowledged.

I thankfully acknowledge Shri. V. Ravindranathan and Dr. V.N. Sanjeevan, Former Directors of CMLRE, Ministry of Earth Sciences for providing financial supports as Junior and Senior Research Fellowships under the project "Bioactive compounds from marine microorganisms". I also gratefully acknowledge Dr. T.V. Raveendran, Scientist, NIO, Kochi for fruitfully mentoring my research orientation and outlook.

I place on record the camaraderie and support rendered by my dear friends, Dr. Padmakumar K.B., Dr. Sanilkumar M.G. and Dr. Anit. M. Thomas. The well-planned and extensive field trips executed along with them are indeed the backbone of this thesis work. Their company and friendship made my years at the university even more wonderful. The warmth and goodwill of my dear friends Dr. Harisankar H.S. and Mr. Vijayakumar P. are beyond words. Their tireless efforts have been a constant energy for me through this long journey. I am deeply obliged to my former colleague and dear friend, Sanu V.F., for his selfless love and support in making this work possible.

I would also like to offer my heartfelt thanks to Dr. M.P. Prabhakaran, Asst. professor, KUFOS, for always being there as a pillar of strength. The warm and valuable friendship of Dr. Anas Abdulazeez, Scientist, NIO, is deeply cherished. His words have always been a source of fresh energy. I express my deep sense of gratitude to Dr. Laluraj C.M., Scientist, NCAOR, Goa, who provided a constant source of encouragement and support in my efforts.

I am extremely grateful to Dr. Nandini Menon, Dr. Valsamma Joseph, Dr. Sunesh Thampy, Dr. Selven S., Dr. Sajeevan T.P., Dr. Neil Scolastine Correya, Dr. K.P. Krishnan, Dr. Johnson Zacharia, Dr. Baiju K.R., Dr. Baiju K.K, Dr. Shaiju P., Dr. Deepulal P.M., Dr. Gireeshkumar T.R., Dr. Jayesh P., Dr. Prajith K.K., Mr. Anil Kumar P.R., Dr. Abdul Jaleel, Dr. Najumudeen T.M., Dr. Smitha C.K., Dr. Simi Joseph, Dr. Priyaja P., Dr. Manjusha K., Dr. Deepthi Augustine, Dr. Reema Kuriakose, Mr. Shubankar Ghosh, Dr. Ramya Varadarajan, Dr. Jisha Jose, Ms. Anila T.N., Dr. Maya Paul, Dr. Lakshmi G. Nair, Dr. Meera Venugopal, Dr. Harikrishnan E. and Dr. Swapna P. Antony for all their support and goodwill during my tenure at the University.

The patience and support of my parents, Mr. Rajendran Nair and Mrs. Sreedevi Nair and in-laws, Mr. Padmanabhan Nair and Mrs. Valsala cannot be expressed in words. Their whole-hearted support was indeed my driving force to complete the programme. My wife, Limna was all through with me, through the thick and thin of this wonderful journey. Her faith was my motivation that has led me to successfully compile this thesis. Above all, I am indebted to my kids, Avanthika and

Shrihan for their innocent cooperation throughout the period of my doctoral programme.

I express my gratitude to all those who have been a part of this journey, at one point or the other.

To God be the glory……For he has done.

Abhilash K.R.

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List of Abbreviations

%	:	Percentage
°C	:	Degree Celsius
µl	:	Microliter
bp	:	Base Pair
G P	:	Gram Positive
G N	:	Gram Negative
H’	:	Shannon Wiener Diversity
J’	:	Pielou’s Evenness
d	:	Species Richness
wt.	:	Weight
v/v	:	Volume by Volume
w/v	:	Weight by volume
DF	:	Degrees of Freedom
et al.	:	*Et alli* (Latin word, meaning "and others")
cfu	:	Colony Forming Units
G+C	:	Guanine+Cytosine
mM	:	MilliMolar
OM	:	Organic Matter
pH	:	Hydrogen Ion Concentration
SD	:	Standard Deviation
MW	:	Molecular Weight
$g\ l^{-1}$	:	Gram per Liter
$mg\ g^{-1}$	:	Milligram per Gram
rpm	:	Revolution per Minute
psu	:	Practical Salinity Unit
PRT	:	Protein
LPD	:	Lipid
CHO	:	Carbohydrate
TOC	:	Total Organic Carbon

LOM	:	Labile Organic Matter
TOM	:	Total Organic Matter
BPC	:	Biopolymeric Carbon
DNA	:	Deoxyribonucleic Acid
POM	:	Particulate Organic Matter
THB	:	Total Heterotrophic Bacteria
PCR	:	Polymerase Chain Reaction
MDS	:	Multi Dimensional Scalling
DOM	:	Dissolved Organic Matter
dNTP	:	Deoxyribonucleotide Triphosphate
MoES	:	Ministry of Earth Sciences
rDNA	:	Ribosomal Deoxyribonucleic Acid
EDTA	:	Ethylene Diamine Tetra Acetic Acid
Tris HCl	:	Tris(hydroxymethyl)aminomethane Hydrochloric acid
ANOVA	:	Analysis of Variance
BOD	:	Biological Oxygen Demand
Chl a	:	Chlorophyll a
DO	:	Dissolved Oxygen
MON	:	Monsoon
NO_2	:	Nitrite
NO_3	:	Nitrate
PO_4	:	Phosphate
POM	:	Post-monsoon
PRIMER	:	Plymouth Routines in Multivariate Ecological Research
PRM	:	Pre-monsoon
sp.	:	Species
spp.	:	Species complex
SPSS	:	Statistical Package for the Social Sciences
SW	:	Southwest

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CHAPTER 1 - GENERAL INTRODUCTION

1.1. Introduction

The ocean is a storehouse of unknown facts. It covers 70% of the earth's surface and is inevitably the largest ecosystem supporting a multitude of living organisms ranging from the minute microbes to the mega mammals. The microorganisms are the key to life in the oceans. They play a major role in the diagenesis of bottom sediments by participating in many chemoautotrophic and mixotrophic reactions in sediments, thereby mediating organic matter re-mineralization (Zobell, 1938; Berner, 1980). Marine microbes thrive not only in the surface waters of the sea, but also in the lower and abyssal depths from coastal to the offshore regions, and from the general oceanic to the specialized niches like blue waters of coral reefs to black smokers of hot thermal vents at the sea floor (Qazim, 1999).

1.2. Coastal Environment

The coastal marine environment is a dynamic system. It harbors a rich and varied diversity of living forms. The physical processes such as brief to prolonged wind-driven circulation and the geological processes like sea level changes force natural fluctuations in the biological communities of the coastal ecosystems (David-Omiema and Ideriah, 2012). The majority of the world's populations are concentrated in the major cities situated along the shorelines. The past few decades have witnessed an exponential rise in population density and subsequent increase in anthropogenic activities. The coastal belt is facing a rush of rapid urban and industrial development. The coastline of India has several expanding towns and industrial centers. The major pressures inflicted by this development on the coastline are release of untreated sewage and municipal waste, potentially introducing pathogens and causing eutrophication in the near-shore waters; agricultural / aquaculture runoff depositing toxic pesticides, fertilizers and pharmaceutical

compounds in the aquatic system, which in-turn results in eutrophication and algal blooms; and industrial development along the coast resulting in oil spills and pipeline leaks, ship ballast water release, chemical plant effluent discharge, sea cargo vessel traffic and warmed high salinity power plant outfalls, increasing risks to seabirds, mangroves and fisheries. The unscientific land-use pattern has thus altered the coastal environment to the extent of enhanced nutrient loading along with trace metal and carbon. In addition, there is an apparent increase in sedimentation rate in coastal ecosystem (McLaughlin, 2000). Dramatic and sudden increases such as blooms, shifts, die-offs in components of the biological communities etc. have been related to anthropogenic perturbations. Such unpredictable phenomena may be expected to continue and to intensify as human populations along the shoreline increase (Verschueren, 1983; Balba and Bewley, 1991; Capone, 1992).

Coastal plains and seas include the most taxonomically rich and productive ecosystems on the earth. Among these, mangrove forests are well over 20 times more productive than the average open ocean and estuaries, salt marshes and coral reefs have 5-15 times higher and shelf seas and upwelling zones 2-5 times higher productivity. These enhanced rates of primary production result in an abundance of other life forms, including many species of commercial importance. Coastal shelf seas yield 90% of the total marine catch of fish, crustaceans and edible molluscs. Further, the coastal zone is also a dynamic area with many cyclic and random processes owing to a variety of resources and habitats. Nearly three quarters of the world population lives on the coast and is found to be true in India also. The coastal region is thus a place of hectic human activity, followed by intense urbanization, resulting in human interference of rapid development. The coastal ecosystems are now highly disturbed and very much threatened, encountering problems like pollution, siltation and erosion, flooding saltwater intrusion, storm surges and other activities due to ever expanding human settlements. Such developments could result in adverse effects on the native species, including the microbiota and subsequently impact the nutrient cycling of the marine environment.

Indian mainland has a coastline of 5,717 km with many sprawling and still growing coastal sites. In the west coast of India, Kerala is the largest coastline with

590km length. The coastal belt of Kerala is interspersed with mangroves, estuaries and coastal plains. The mangroves are distributed in Keeryad Island, northern part of Kochi Port and Research Farm at Puthuvypu, Mahe to Dharmadam coastal belt, Mallikkad, Ashram, Pathiramanal, Mangalavanam and in several other small bits. It is reported that 17 true mangrove species and 23 semi-mangrove species occur in the State (Unni and Kumar, 1997). The major estuaries of the State are Ashtamudi, Korapuzha, Beypore and Periyar. The texture of the coast north of Kozhikode and south of Kollam is mainly rocky but at certain places sandy beaches are formed especially at bay-heads and river confluences. The central part of Kerala coast is mainly sandy.

1.3. Coastal microbiota

The microorganisms in the coastal waters include bacteria, fungi, algae, protozoa, rotifers, crustacean, worms, bacteriophages and insect larvae (David-Omiema and Ideriah, 2012). The sediment bacteria are especially important since they comprise a major fraction of the total benthic biomass, contributing significantly to the turnover of organic matter within the sediments (Billen *et al*., 1990; Deming and Baross, 1993; Kuwae and Hosokawa, 1999). Studies indicate that sediment bacteria are more capable of degrading organic matter as compared to their counterparts in the water column (Sinkko *et al*., 2013). This can possibly be attributed to the relatively harsh conditions in the sediments as compared to the water column. The organic matter together with various anthropogenic contaminants tends to bioaccumulate in the sediment. Also, the particulate organic matter reaching the water finally sinks to the sediment layer. The organic matter accumulating in the sediment as detritus consists primarily of proteins and carbohydrates, followed by lipids in small quantities. The sediment bed has an upper aerobic layer which is only a few millimeters thick, followed by an anaerobic layer. The bacteria harboured in the sediment-water interface play a significant ecological and biogeochemical role in marine ecosystems due to their high abundance relative to the overlying water column and their role in the degradation of organic matter, nutrient cycling and carbon flux (Thiyagarajan *et al*., 2010).

Organic matter in sediment consists of carbon and nutrients in the form of carbohydrates, proteins, fats and nucleic acids. Bacteria quickly consume the less resistant molecules, such as the nucleic acids and many of the proteins. Sediment organic matter is derived from plant and animal detritus, bacteria or plankton formed *in situ*, or derived from natural and anthropogenic sources in catchments. Sewage and effluent from food-processing plants, pulp and paper mills and fish-farms are examples of organic-rich wastes of human origin. Total Organic Carbon (TOC) refers to the amount of organic matter preserved within sediment. Sediment nutrients are assessed as Total Nitrogen (TN) and Total Phosphorus (TP) concentrations, and have inorganic as well as organic sources. The amount of organic matter found in sediment is a function of the amount of various sources reaching the sediment surface and the rates at which different types of organic matter are degraded by microbial processes during burial. The coastal environment contains a mixture of microorganisms capable of metabolizing organic matter, including aerobic heterotrophs and chemolithotrophs such as, hydrogen-oxidizing bacteria, sulphur-oxidizing bacteria, iron-oxidizing bacteria, nitrifying bacteria, nitrate-respiring bacteria, metal-respiring bacteria, sulphur and sulphate-reducing bacteria, methanogens, acetogens, methanotrophs and syntrophic bacteria (Zhang *et al*., 2008). Heterotrophic bacteria, in spite of their minute size, are highly significant in pelagic and benthic processes. They actively break down organic carbon, utilizing electron acceptors, and transform a major fraction of the metabolized organic matter into cell material (Froelich *et al*., 1979). The varied roles of heterotrophic bacteria include utilization of labile fraction of dissolved organic matter (DOM), microbial loop and cycling of bio-essential elements. In the marine environment, they are able to increase or decrease their activity over wide ranges of chemical and physical settings than any other group of organisms. In a typical heterotrophic system, bacteria decompose the organic compounds, utilising oxygen in the due course (Abhilash *et al*., 2012). Heterotrophic bacterial degradation promotes organic material transformation and mineralization processes in sediments and in the overlying waters. They breakdown complex organic substances into simpler fractions, releasing dissolved organic and inorganic substances. The major part of the carbon flow is, therefore, channeled through the bacteria and the benthic microbial loop (Danovaro

et al., 2000). Thus, heterotrophic bacteria are the major agents in shaping the organic composition of the ocean (Raghukumar *et al.,* 2001).

Due to their high turnover rate and metabolic activity, the structure of microbial assemblage is sensitive to changes in trophic conditions (Hansen and Blackburn, 1992). Bacterial communities are structured by temporal and spatial variability of physicochemical and biotic parameters (Hewson *et al*., 2007). They respond to environmental and pollution changes at extremely faster rates. The microbiota, thus, reflects their micro environmental conditions and transfers this information to other biota in their vicinity and play a key role in benthic-pelagic coupling. The estimation of bacterial abundance as well as their genetic diversity under in situ conditions is therefore the most fundamental objective of aquatic microbial ecology (Thiyagarajan *et al*., 2010). The microorganisms have the capability to adjust to varying organic loads and environmental influences such as temperature, salinity, hardness, pH etc. in the ecosystem. However, extreme temperature, high concentrations of toxic metal ions or toxic chemicals can decrease or exterminate the activity of the microorganisms. In order to better understand heterotrophic bacteria and their processes in the marine environment, a perception on their abundance, distribution, production and their involvement in nutrient cycling and food web is essential.

1.4. Marine microbial enzymes

The diversity and qualitative structure of bacteria capable of mineralizing organic matter is less explored. Whether the entire spectrum of heterotrophic bacteria or only selected groups is involved in the process is still not clear (Martinez *et al*., 1996). The tools used by bacteria for organic matter degradation is the factory of enzymes harboured by them. Based on the quantum of organic matter available in the environment, the bacteria are capable of regulating the synthesis and activity of hydrolytic enzymes, resulting in the release of simple monomers. Fundamentally, all enzymes are protein molecules with catalytic properties and potential for specific activation (Komberg, 1989). Activity of an enzyme depends upon various parameters including enzyme concentration, substrate concentration, pH, temperature as well as

the presence of inhibitors and co-factors. The sustenance of benthic ecosystems largely depends on the input of organic material, mostly in the form of polymeric organic compounds. These compounds are then decomposed by extracellular enzymes secreted by the bacterial cells and the resulting simpler compounds are subsequently utilized by the bacteria for their energy and biomass requirements (Unanue *et al.,* 1999). Many heterotrophic bacteria have been reported to carry genetic and metabolic potentials to synthesize and control extracellular enzymes, which can degrade and modify a large variety of natural polymers in the marine environment (Munster and Chrost, 1990; Mudryk and SkoRczewski, 2004).

Over the years, the enzyme-producing potential of marine microorganisms has been utilized by man for a variety of processes. Marine microorganisms, being easy to isolate, maintain, identify and bioprocess, have been of major interest to enzyme researchers worldwide. They are a rich source of enzymes and bioactive compounds. Bioactivity can be antibacterial, antifungal, antiviral, anti-inflammatory, anticancerous, antibiotic, anticoagulant, hormones, narcotics and vitamins. The vast array of microbial extracellular enzymes include proteases, lipases, amylases, peptidases, glucomylases, invertases, malt-diastases, lactases, α-galactosidases, cellulases, hemicellulases, pectinases, chitinases, phytases, phosphatases, arylsulfatases, L-asparaginases, L-glutaminases, ureases, lactamases etc. These extracellular enzymes have numerous applications in food, dairy, pharmaceutical, agricultural, cosmetic and detergent industries. Microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Enzyme production is closely controlled in microorganisms, and therefore, to improve its productivity these controls can be exploited and modified. Further, the occurrence in extreme environments like hydrothermal vents, symbiotic associations with higher organisms like sponges, corals etc. and predominance in marine sediment and seawater envisage the marine microorganisms to be potential sources of novel biocatalysts.

Enzymes isolated from microbes of hydrothermal vents with temperatures of 350-400°C are stable protein molecules with activity for longer time periods compared to regular enzymes. They do not get destabilized by organic chemicals

used in industrial downstream processes (Ghosh *et al*., 2005). Thermostable polymerase chain reaction (PCR) enzymes isolated from bacteria living near hydrothermal vents has already been marketed as Vent and Deep Vent polymerases Likewise, psychrophilic enzymes are also useful ingredients of commercial detergents, since washing can be done in cold instead of hot water, thereby reducing power consumption. Some of the novel enzymes isolated from extremophiles are the hyperthermophilic, barophilic protease from *Methanococcus jannaschii* (Michels and Clark, 1997), the intracellular serine proteinase (pernilase) from the aerobic hyperthermophilic archaeon, *Aeropyrum pernix* (Chavez *et al*., 1999), the NAD(P)-dependent dehydrogenases from the Antarctic psychrophile, *Cytophaga* sp. (Soda *et al*., 2002) etc. Proteases, carbohydrases and peroxidases have been the most reported enzymes from near-shore sediments, deep sea sediments and seawater.

From the ecological perspective, microbial enzymes play an important role. Microbiological processes in coastal areas contribute to ecosystem changes on a large scale. Soil microorganisms play a significant role in the food chain and the various biogeochemical cycling of carbon, nitrogen, sulphur and phosphorus (Kummerer, 2004; Banig *et al*., 2008). Where human induced changes have been adjudged to be negative, the natural micro biota or introduced surrogates may be a useful means for some level of restoration through bioremediation. Therefore, estimating the microbial community structure in sediments from the continental shelf (i.e. the zone most exposed to pollutants) is essential to understand microbial processes underlying secondary pollution phenomena. Moreover, harnessing the enzyme potential harboured by the marine microbes will be useful in formulating various natural catalyzing agents.

1.5. Scope of the study

Marine sediments form the largest microbial habitat. Studies on biodiversity and its relation to ecosystem structure and function have mainly focused on macroorganisms, and little attention has been directed towards microorganisms. Bacteria in sediments include aerobic heterotrophs and chemolithotrophs. Sediment bacteria play a significant ecological and biogeochemical role in marine ecosystems

due to their high abundance relative to the overlying water column. They play a key role in the decomposition of the organic matter, nutrient cycling and carbon flux. Despite their importance, our knowledge of the bacteria that inhabit surface sediments is limited, especially in the heterogeneous marine ecosystems. Estimation of bacterial abundance and their diversity is essential for the understanding of the aquatic microbial ecology. The detection of bacterial diversity and their spatio-temporal variation in surface sediments is also of great practical and scientific relevance, especially in coastal ecosystems. Their biodiversity is structured and determined by the temporal and spatial variability of physicochemical and biotic parameters and thus, can reflect local environmental conditions. Shift in nutrient, environmental and pollution profiles in the benthic-pelagic ecosystems will directly impact bacterial community that in turn further affects nutrient cycles and other related communities. They are vulnerable to natural and anthropogenic disturbances such as global climate change, pollution, heavy metal contamination, organic pollution and enrichment.

Bacteria from marine environment secrete different enzymes based on their habitat and their ecological functions. It has become the focal point of interest with several enzymes being isolated, purified and characterized for their properties and application. Several industrial enzymes derived from marine organisms are yet to be exploited to the full potential and thus marine microbes are of interest for microbial enzymes. The microbial extracellular enzymes catalyze reactions in the mineralization processes and cycling of elements in environment. Thus microbes form a dependable source of enzymes such as protease, amylase, lipase, chitinase, cellulase, ligninase, pectinase, xylanase and nucleases. The roles of these enzymes are to breakdown the complex organic matter reaching the benthic realm through degradative pathways of their metabolism. Complex polysaccharides like cellulose, lignin, pectin, xylan and starch along with various proteins, fats, sugar, urea, aromatic and aliphatic hydrocarbons reach the sediments. Heterotrophic bacteria living in particle aggregates and sediments depend on extracellular enzymes to generate low molecular weight compounds (<600 Daltons) from these complex molecules for uptake and metabolism.

Among the various enzymes isolated from marine microbes, lipases are the catalytic agents of hydrolysis, alcoholysis, acidolysis, esterification and aminolysis, making them inevitable for industrial applications (Hasan *et al*., 2006). An important aspect of lipases is that they do not require cofactors for catalysis (Macrae and Hammond, 1985). Due to their lipolytic as well as esterolytic activity, lipases are able to utilize a wide range of substrates. This, along with their high stability towards a wide range of temperature, pH and organic solvents, makes lipases one of the most important biocatalysts in the present day world (Gupta *et al*., 2004; Ferreira-Dias *et al*., 2013).

In the current study, the composition and variability of heterotrophic bacteria across various seasons along the coastal habitats of Kerala were investigated for a period of two years. Their ability to produce various extracellular enzymes was also studied and the partial purification of alkaline lipase from a dominant strain was undertaken.

1.6. Objectives of the study:

- Evaluation of the general hydrography and sediment properties of the coastal waters along Kerala, south west coast of India.
- Quantitative and qualitative assessment of the total heterotrophic bacteria (THB) in the coastal waters of Kerala, south west coast of India.
- Assessment of the generic composition of heterotrophic bacteria isolated from the coastal waters of Kerala, south west coast of India.
- Determination of the extracellular hydrolytic enzyme production potential of the bacterial isolates.
- Isolation and characterization of the lipase enzyme produced by the most potent strain.

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CHAPTER 2
HYDROGRAPHY AND SEDIMENT CHARACTERISTICS

2.1. Introduction

The marine ecosystem consists of the pelagic and benthic realms comprising the water column and the sediment matrix respectively. These two are surprisingly distinct but entirely interdependent compartments (Bacci *et al*., 2009). Marine sediments constitute the single largest ecosystem on earth in terms of spatial coverage wherein the benthic compartment extends from intertidal region up to the deepest trenches (Snelgrove, 1998). The benthic substratum is considered to be a location of remineralization and burial of organic carbon (Walsh *et al*., 1985). The coastal waters are important as they are rich in biodiversity and as a support source for the livelihood for coastal population reliant on the coast particularly the fishing communities. Approximately 98% of all marine organisms are assumed to belong to the benthic community (Peres, 1982). It is generally termed as phyto-benthos for plants, zoo-benthos for animals and micro-benthos for sediment associated microorganisms, which include viruses, bacteria and fungi communities. They are important as they are responsible for the recycling of the organic matter that ultimately arrives in the marine realm for their metabolic purposes. The tropical coastline is regarded as a complex ecosystem with diverse habitats viz. sea grass beds, coral reefs, mangrove swamps, creeks, deltas and bays. This system is influenced by a wide range of physical (meteorological and oceanographic) and biogeochemical factors.

Coastal zone is considered to be one of the most rich and productive ecosystems. India has a coastline of 5,423 km (Kumar *et al.,* 2006) (excluding the islands) in which Kerala coast is highly significant for the unique physiographic setting which is responsible for the environmental variability and dynamism. However, recent studies indicated that various factors including excessive anthropogenic influences has impacted the community structure of metabolically

active microorganisms, which in turn influences the functioning of coastal food webs and general biogeochemical ecosystem. These vibrant ecosystems are considered as threatened by the development associated concerns due to expanding human settlements like pollution, siltation, erosion and storm surges (Manju & Sujatha; 2012). The west coast is environmentally more vulnerable compared to the east coast of India as the Arabian Sea being rich in its biological production throughout the year in the course of different processes (Mathupratap *et al*., 1996). Compared to any other coast of the developed world this too is a highly sensitive ecosystem serving as reservoirs for dredged sediments due to developmental activities, sewage due to high congregations of human settlements, industrial and municipal effluents and various types of natural and terrigenous pollutants. Near shore coastlines are the critical land–ocean interfaces marked by the anthropogenic (Baldwin *et al.,* 2005) and terrestrial fluxes that are characterized by naturally derived organic matter (autochthonous production) from the open sea, contiguous salt marshes and river drainage (Andrade *et al.,* 2003; Giovannoni *et al.,* 2005). Kerala coast has exerted tremendous pressure on the natural coastal ecosystem with its beautiful beaches, estuaries and lagoons, due to the very high (~2362/km^2) population density.

Microbial communities consist of viruses, eubacteria, archaebacteria, fungi, protozoa, and algae (Kemp *et al.*, 1990). They are universally distributed in marine systems and eubacteria make up significant components of the ecosystem (Wikner *et al.,* 1999). The study on microbial communities of coastal ecosystem is a pre-requisite as it addresses the characteristics and roles of microorganisms in natural environment (Pomeroy and Wiebe, 1991). Biologically dynamic ecosystems show high biomass of heterotrophic bacteria as they are responsible for the bulk of organic carbon transformation as it is utilized for respiration in the sea. Their roles in nutrient and energy fluxes are crucial for the functioning of marine ecosystems and often dominate the biomass of microbial food webs (Rappé and Giovannoni, 2003). Over the past few decades, microbial ecologists have been examining the microbes and processes involving the biogeochemical processes of nutrient cycling. Particulate organic matter from the water column sinks through and eventually reaches the ocean floor and forms a potential source of food for benthic organisms. Marine sediments are considered as the burial site of organic matter thus forming the largest

reservoir on earth (Cowie, 2005). Sinking of the organic matter through the water column is essentially a physical process, once the organic matter reaches the bottom; its fate is decided by the benthic organisms either feeding by microorganisms or the degradation by microorganisms. Autochthonous and allochthonous organic carbon from a variety of sources are released for biogeochemical cycles by the combined action of various microorganisms (Strom *et al.*, 1997). This process is mainly dictated by the hydrolytic potential of metabolically active microorganisms, which secretes various extracellular enzymes and degrades polymeric particulate organic matter into dissolved organic matter (Peduzzi and Herndl, 1992; Conan *et al*., 1999). Microbes of the ocean floor are the major consumers of organic matter thereby responsible for its transformation and mineralization for the recovery of organic matter from detritus to living biomass (Ducklow and Carlson, 1992; Shiah and Ducklow, 1994).

For the coastal sediments the major factors which control distribution of benthic bacterial population include physical characteristics such as temperature, light, salinity, dissolved oxygen, pH, hydrostatic pressure, water movements and sediment type (Deming and Baross, 1993; Bak and Nieuwland, 1997). For mangrove stations in addition, to these the varied periods of inundation and evaporation makes the habitat a very unique in the sense that only tolerant forms of organisms, right from microorganisms to mangroves will only be able to thrive and survive here. Like other benthic inhabitants, bacteria in shelf sediments are related to the sediment properties.

2.2. Materials and Methods

2.2.1. Description of the Study Region

2.2.1.1. Nearshore coastal regions of Kerala

Based on the geographic distinctiveness the west coast of India is divided into Kuchchh, Saurashtra, Konkan and the Malabar coasts. The Malabar Coast where the State of Kerala situated is distinguished by a chain of brackish water lagoons and lakes lying parallel to the coast. The network includes five large lakes linked by canals, both manmade and natural, fed by 38 rivers, and extending virtually half the length of Kerala state with 34 backwater systems (Mathew, 1991). The Vembanad

lake, south of Kochi is the largest one followed by Ashtamudi lake further south. The total length of Kerala coast is 590 km, which is about 10% of the total coast length of India. 83.93% of this coast is affected by erosion with 15% of the coast being muddy and 5% of rocky and an 80% of sandy substratum. The wave height varies from 0.3-1.9 m, with a sediment transport ranging from 12.66×10^5 m^3/year and 5.99×10^5 m^3/year during northerly and southerly respectively. The Malabar Coast is considered as submerged and dominantly non-rocky type with estuaries, cliffs, spits, lagoons, beaches as the prominent geomorphologic features (Kumar *et al.,* 2006). Geomorphologically, Kerala coast can be classified into two categories, rocky and sandy. The coasts north of Kozhikode and south of Kollam are mainly rocky but at certain places sandy beaches are formed especially at bay-heads and river confluences. The central part of Kerala coast is mainly sandy.

The estuaries or backwaters were formed by the action of waves and shore currents creating low barrier islands across the mouths of the many rivers flowing down from the Western Ghats range. In spite of so many rivers discharging into the sea, no major delta has been formed anywhere. Greater part of the shoreline of Kerala is straight i.e., from Kozhikode to Kollam, but in Kannur, Thiruvananthapuram and Kollam districts, indentations, cliffs and protuberances are present. The Tertiary sedimentary cliffs in Varkala are a unique geological feature of the otherwise flat Malabar. The shoreline is a compound one with a variety of features some of which have resulted from submergence and others from emergence. The coastal plain from Alapuzha to Kochi has a series of parallel to sub parallel sand dune ridges. Sea erosion on the coastal tract is a frequent feature of Kerala. But now groins and seawalls serve as a protection against sea erosion.

The annual rainfall is high ranging from 200-400 cm most of which falls during the south-west monsoon (Simon and Mohankumar, 2004). During the north-east monsoon the rainfall is negligible. The climate is tropical with three seasons as (1) Monsoon from June to September, (2) Post-Monsoon from October to January (3) Pre-monsoon from February to May. The tides are semi-diurnal type with the coastline being low and frequent flooding of coastal areas by storm tides in many areas during the south-west monsoon. The sea becomes rough during the monsoon

months (June - September) with high waves of average height of 3.2 m, and wave periods of 5-12 seconds amid storm surges, attack the coast (Mathew, 1991).

2.2.1.2. Mangrove Stations along Kerala coast:

Primarily mangrove ecosystems are reasonably diverse, and are habitat for several endangered species. They are physiologically unique in their ability to live in salt and brackish water. The mangrove communities form important stabilizers of fine sediment particles with a substantial amount of sediment being deposited with each retreating tide. The associated algal forms on the sediment surface help in binding the sediment particle together thereby acting as a buffer against coastal erosion (Sebastian, 2002). Mangrove, and the sediments associated with them, can assimilate substantial quantities of nutrients thereby preventing contamination of nearshore waters and may reduce the incidence of eutrophication (Robertson *et al.,* 1992).

In Kerala, mangrove forests that once occupied about 700 km^2, have now dwindled to 17 km^2. As in many other parts of the world, the vegetation has diminished in its extent drastically and has acquired a ‘threatened’ status in Kerala (Ramachandran *et al.*, 1986; Basha, 1991). The mangroves which once fringed the backwaters of Kerala have now been reduced to a few isolated patches consisting of a few species. Of the 14 Districts in Kerala, mangroves are spread over in 10 Districts. Kannur has highest area under mangroves (755 ha), followed by Kozhikode (293 ha) and Ernakulum (260 ha). Thus Dharmadom in Kannur, Kadalundi in Kozhikode district and Puthuvaipu in Ernakulam district where the major mangrove areas are concentrated, were considered for the study. Puthuvaippu mangrove forest is directly connected to sea through a canal. It is a sea accreted landform considered to have been formed after 1929 since the opening of the bar-mouth of Cochin. *Avecennia officianalis* and *Bruguiera gymnorrhiza* are the dominant mangrove species in this area of about 20 ha of mangrove patches with several tidal channels, sand pits and creeks supporting the growth of mangrove vegetation (Sebastian and Chacko; 2006; Rejil, 2012). Patches of mangrove species are distributed near Dharmadam Island, which is surrounded by Dharmadam estuary. In Kannur district

total mangrove area had been estimated to be 9.47km^2. Kannur district occupies the highest extent of mangroves in the State with more than 60 per cent of the total mangrove areas under private ownership (FSI, 2003). Mangroves are luxuriant in certain areas due to the absence of development with diversity of pure mangroves very high when compared to other districts. This region had undertaken extensive mangrove afforestation programmes under the auspices of Department of Forest, Government of Kerala. Forest Survey of India (FSI, 2003) further showed that mangrove vegetation in Kerala is now restrained largely to river mouths and tidal creeks and that there has been no significant mangrove cover south of Cochin in Kerala coast. Kadalundi, is an estuarine cum mangrove area located in Kozhikode district wherein the estuarine marshland area displays the functional characteristics and role of a mangrove wetland system. During low tide, as the tidal flood waters recede, the open areas of the estuary are exposed up to the eastern end (Shamina *et al.,* 2014). The mangroves and the mangrove wetland system in and around Kadalundi offer congenial habitats for many fauna including migratory birds (Vidyasagaran *et al*., 2011)

2.2.2. Sampling strategy

Seasonal sampling was undertaken along three coastal stations, four estuarine stations and three mangrove stations during March 2006 (Pre Monsoon), August 2006 (Monsoon), January 2007 (Post-Monsoon), April 2007 (Pre-Monsoon), August 2007 (Monsoon) and January 2008 (Post-Monsoon). The stations were fixed on the basis of specific geographical features, water flow regimes and anthropogenic activities (Fig.1, Table 1).

Fig.1. Sampling stations along the Kerala coast

Table 1. Characteristics of the sampling locations

Sl No.	Station	Habitat	Latitude	Longitude
1	Kodikal	Coastal	11°28’43” N	75°36’10” E
2	Punnapra	Coastal	09°25'23" N	76°19'41" E
3	Vaadi	Coastal	08°52'01" N	76°34'26" E
4	Mahe	Estuary	11°42'18" N	75°32'36" E
5	Balathuruth	Estuary	11°07'50" N	75°49'57" E
6	Azhikode	Estuary	10°11'02" N	76°09'22" E
7	Fort Kochi	Estuary	09°58’12”N	76°13’53”E
8	Kavanad	Estuary	08°55'55" N	76°33'37" E
9	Dharmadom	Mangrove	11°47’32” N	75°27.41” E
10	Kadalundi	Mangrove	11°07’58” N	75°50’25” E
11	Puduvaippu	Mangrove	09°59’49” N	76°13’36” E

2.2.3. Hydrographic parameters

Water samples for determining physicochemical parameters were sampled using Niskin sampler and further collected in polypropylene bottles and preserved in ice for analysis. General hydrographical parameters and nutrients of the bottom waters were analyzed using standard methods. Major nutrients like nitrate, nitrite, phosphate and silicate of bottom water samples were analyzed in the laboratory using standard procedures. Nitrate was reduced to nitrite using copper-coated Cadmium column and estimated as nitrite (Grasshoff *et al*., 1999). Nitrite was converted to an azo-dye with sulphanilamide and N-(1-naphthyl) ethylene diamine dihydrochloride (Grasshoff *et al*., 1999). Phosphate was analyzed by the ascorbic acid method by formation of phosphomolybdate complex with ascorbic acid as reducing agent was used for phosphate determination (Grasshoff *et al*., 1999). Silicate was estimated by following Strickland and Parsons (1972) by converting it into silicomolybdate complex, which is reduced, using ascorbic acid and oxalic acid, to produce a blue solution. Primary productivity experiments were conducted under in situ condition for three hours by light and dark bottle oxygen method and the values obtained were extrapolated for the day hours (Gaarder and Gran, 1927).

2.2.4. Sediment sampling and geochemical parameters

Sediment samples were collected using a Van Veen grab of mouth area $0.025m^2$ and undisturbed surface sediments were transferred to sterile plastic containers and stored at 4-5°C and analyzed immediately. For biochemical analysis the sediment samples were stored at -20°C. The sediment samples for estimating the textural composition were preserved after drying. The Sediment samples were dried and powdered for estimating organic carbon and labile organic matter.

2.2.4.1. Sediment textural analysis

Sediment samples were analyzed for sand, silt and clay. Each sample was dispersed by stirring in a solution of sodium hexametaphosphate in distilled water overnight, after which higher fraction was removed by sieving (180µm), dried and

weighed. The remaining suspension of fine particles was analyzed using a SYMPATEC H70010 Sucell particle size analyzer (Germany).

2.2.4.2. Elemental composition of sediments

The dried sediment was finely powdered to talc grade and was subsequently analyzed using CHNS analyzer (VarioEL III). The elemental composition of Carbon (C), Nitrogen (N) and Sulphur (S) was expressed as percentage (%).

2.2.4.3. Organic Carbon and Total Organic matter

Sediment organic carbon was estimated by the procedure of El Wakeel and Riley (1956) modified by Gaudette and Flight (1974). The amount of total organic matter (TOM) was obtained by multiplying the organic carbon values with 1.724 (Nelson and Sommers, 1996).

2.2.4.4. Labile Organic Matter (LOM)

The sum of all the sediment proteins (PRT), carbohydrates (CHO) and lipids (LPD) is termed as the labile organic matter (Danovaro *et al.*, 1993; Cividanes *et al.*, 2002). The PRT, CHO and LPD were analyzed separately as discussed below.

i. Estimation of Proteins and Protein Nitrogen in sediment

Protein analyses were carried out following the procedure of Lowry *et al.*, (1951), as modified by Rice, (1982) with albumin as the standard. The amount of protein was expressed as µg/g dry sediment. The amount of protein nitrogen (PN) was obtained by multiplying protein with a factor of 0.16 (Mayer *et al.*, 1986).

ii. Estimation of Carbohydrates

Total carbohydrates were analyzed according to Dubois *et al.,* (1956), using glucose as the standard.

iii. Estimation of Lipids

Total lipids were extracted according to Bligh and Dyer (1959) and estimated according to Barnes and Blackstock, (1973) using Cholesterol as the standard.

In addition to this, the PRT, CHO and LPD concentrations were converted to carbon equivalents by using the conversion factors: 0.49, 0.40 and 0.75 g of C/g, respectively (Fabiano and Danovaro, 1994). The sum of PRT-carbon, CHO-carbon and LPD-carbon is referred to as biopolymeric carbon (BPC) (Fabiano *et al.*, 1995).

2.2.5. Statistical Analysis

The hydrographic and sediment data were analysed by univariate and multivariate statistical methods using the statistical softwares SPSS 16.0 and PRIMER-6 (Clarke and Gorley, 2001). Spatial and temporal variations in environmental variables were examined by two-way analysis of variance (ANOVA). A post-hoc Bonferroni test was adopted to determine if there were significant differences among the seasons. Probabilities (p) of <0.05 were considered to be significant. The independent two-sample (Student's) t-test was carried out to determine significant differences between sampling.

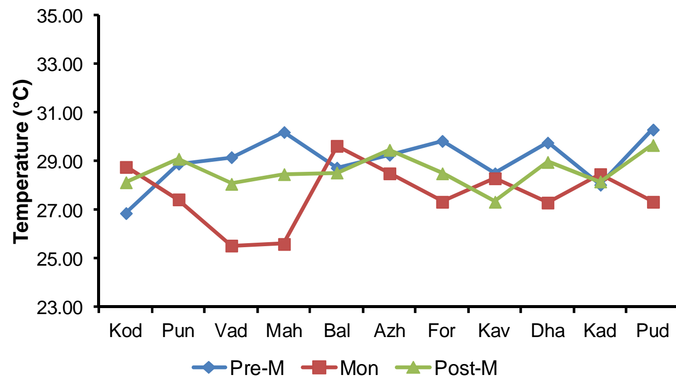
2.3. Results

2.3.1. Hydrography and nutrient parameters

2.3.1.1. Temperature

Both the spatial and temporal variations of temperature were recorded. In 2006-07, the highest temperature of 30.37 ± 0.17°C was recorded during the pre-monsoon period in station 11 and the lowest was recorded as 25.53 ± 0.21°8C during the monsoon at station 3 (Fig.2a). In 2007-08, the highest temperature of 32.3±0.2°C was recorded in station 9 during the pre-monsoon and the lowest was from station 3 in the monsoon period with 25.36 ± 0.2°C (Fig.2b).

(a) 2006-07



(b) 2007-08

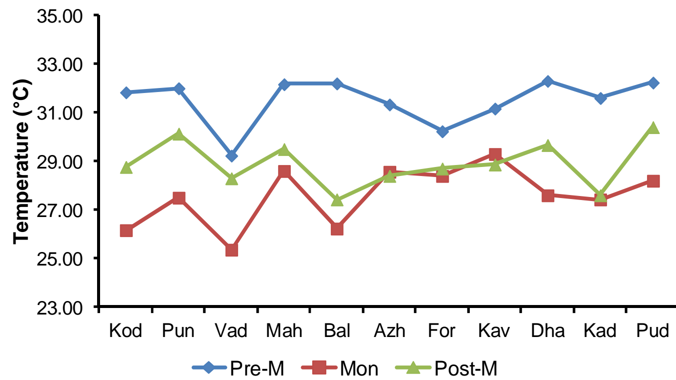


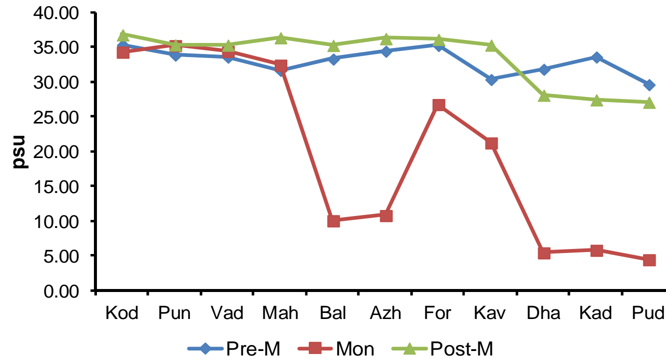
Fig.2. Temperature: spatial and seasonal variations

According to two-way ANOVA, temperature significantly varied with stations ($F_{10,66} = 17.468$, $p<0.001$) and with seasons ($F_{2,66} = 128.977$, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the temperature was not significantly different between the two samplings ($p>0.05$).

2.3.1.2. Salinity

In 2006-07, the highest salinity of 36.8±0.4 psu was observed at station 1, during the post-monsoon and the lowest (7.73 ± 0.11 psu) was recorded at station 6 in the monsoon season (Fig.3a). In 2007-08, the highest salinity recorded was 36.03±0.21psu at station 1 during the pre monsoon and the lowest salinity (6.67±0.61psu) was observed at station 5 during the monsoon season (Fig.3b).

(a) 2006-07



(b) 2007-08

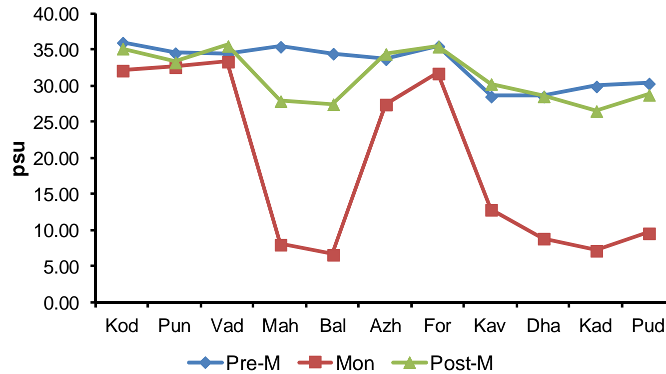


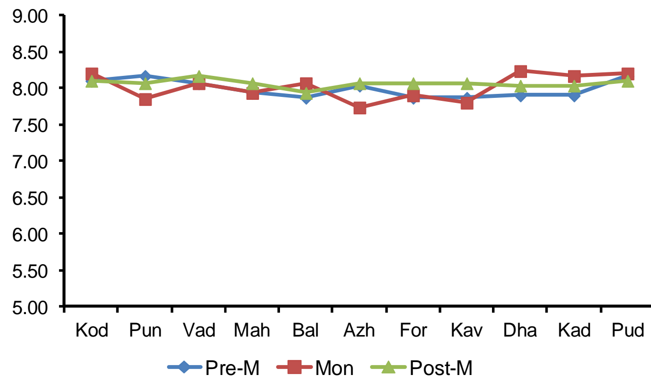
Fig.3. Salinity: spatial and seasonal variations

According to two-way ANOVA, salinity significantly varied with stations ($F_{10,66}$ = 492.370 , $p<0.001$) and with seasons ($F_{2,66}$ = 3267, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the salinity was not significantly different between the two samplings ($p>0.05$).

2.3.1.3. pH

In 2006-07, the pH was maximum (8.23±0.06) at station 9 during monsoon and minimum (7.73±0.11) at station 6 during the same season. The pH remained alkaline in all the stations throughout both the sampling periods (Fig.4a). In 2007-08, the highest pH recorded was 8.3±0.1 at station 6 during the post monsoon and minimum (6.7±0.1) at station 9 during the pre monsoon (Fig.4b).

(a) 2006-07



(b) 2007-08

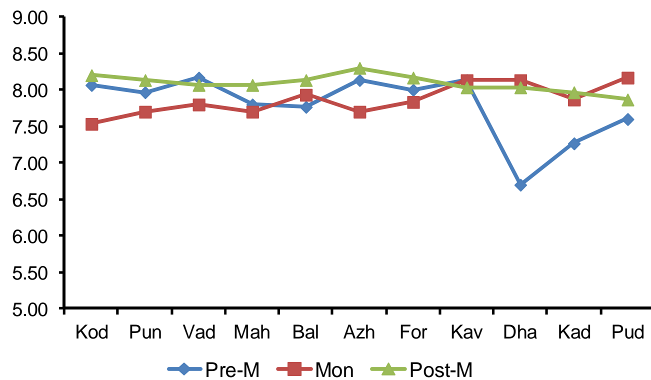


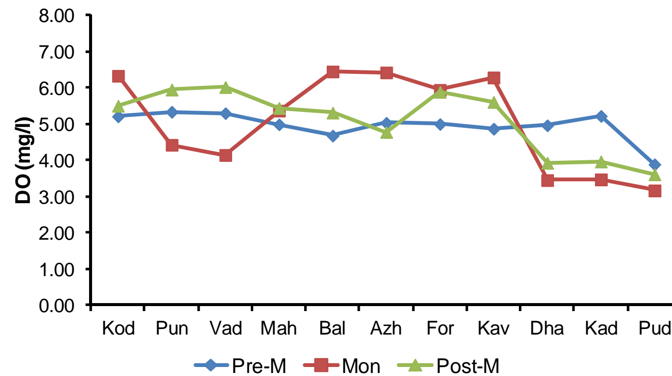
Fig.4. pH: spatial and seasonal variations

According to two-way ANOVA, pH significantly varied with stations ($F_{10,66} = 5.648$, $p<0.001$) and with seasons ($F_{2,66} = 4.499$, $p<0.05$). The result from the independent two-sample (Student's) *t*-test showed that the pH was not significantly different between the two samplings ($p>0.05$).

2.3.1.4. Dissolved Oxygen

In 2006-07, a maximum DO of 6.44±0.55 mgL^{-1} was observed at station 5 and a minimum DO of 3.17±0.19 mgL^{-1} was observed at station 11 during the monsoon season (Fig.5a). Similarly, in 2007-08, a maximum DO of 7.42±0.09 mgL^{-1} was observed at station 8 and a minimum DO of 3.25±0.35 mgL^{-1} was observed at station 11 during the monsoon season (Fig.5b).

(a) 2006-07



(b) 2007-08

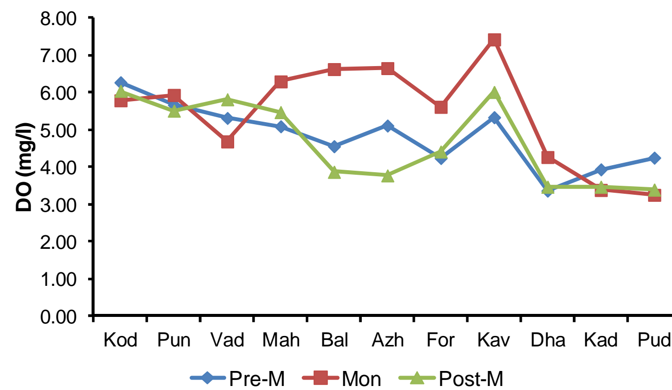


Fig.5. DO: spatial and seasonal variations

According to two-way ANOVA, DO significantly varied with stations (F10,66 = 38.498, p<0.001). However, there was no significant difference in DO between the seasons (F2,66 = 1.325, p>0.05). The result from the independent two-sample (Student's) *t*-test showed that the DO was not significantly different between the two samplings (p>0.05).

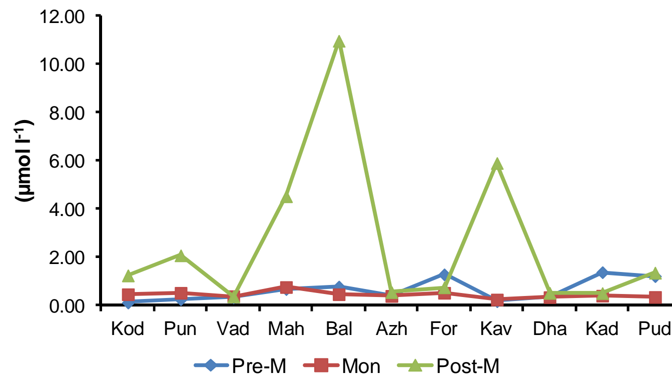
2.3.1.5. Nitrite

In 2006-07, the nitrite concentration was relatively high in almost all the stations during the post monsoon. The maximum NO_2 concentration (10.96±1.42 µmolL-1) was observed at station 5 during post-monsoon, while the minimum (0.12±0.04 µmolL-1) was observed at station 1 during pre monsoon (Fig.6a). In

2007-08, the highest concentration of nitrite (1.52±0.09 $\mu molL^{-1}$) was recorded in station 11 during the pre monsoon period and the minimum (0.14±0.04 $\mu molL^{-1}$) at station 5 during the same season (Fig.6b).

According to two-way ANOVA, NO_2 concentration significantly varied with stations ($F10,66 = 106.929$, $p<0.001$) and with seasons ($F2,66 = 459.814$, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the nitrite concentration was not significantly different between the two samplings ($p>0.05$).

(a) 2006-07



(b) 2007-08

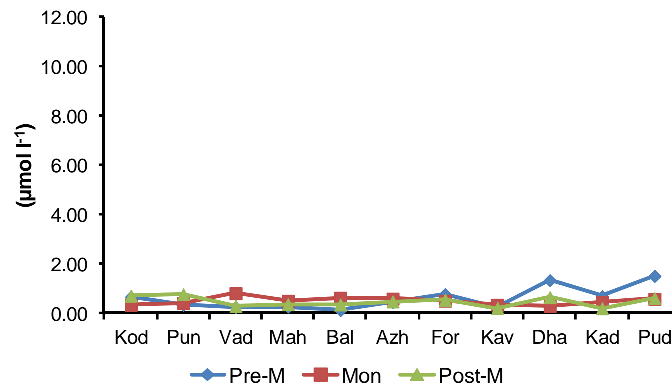


Fig.6. Nitrite: spatial and seasonal variations

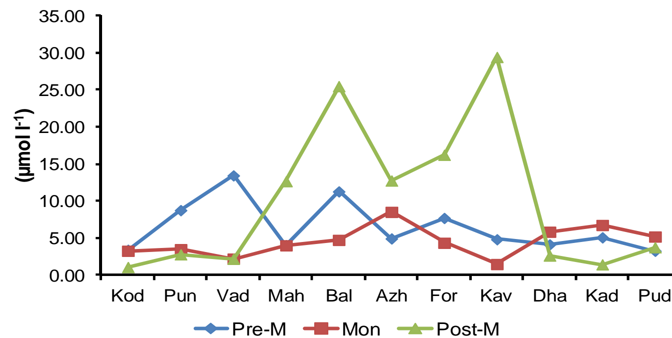
2.3.1.6. Nitrate

In 2006-07, the maximum nitrate concentration (29.4±6.66 µmolL-1) was observed at station 8 during post-monsoon, and the minimum (1.05±0.17 µmolL-1)

was observed at station 1 during the same season (Fig.7a). In 2007-08, the maximum nitrate concentration (13.47±1.08 µmolL-1) was observed at station 7 during pre monsoon, and the minimum (0.81±0.07 µmolL-1) was observed at stations 2 and 5 during the post monsoon period (Fig.7b).

According to two-way ANOVA, NO_3 concentration significantly varied with stations ($F_{10,66}$ = 106.929, $p<0.001$) and with seasons ($F_{2,66}$ = 459.814, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the nitrate concentration was not significantly different between the two samplings ($p>0.05$).

(a) 2006-07



(b) 2007-08

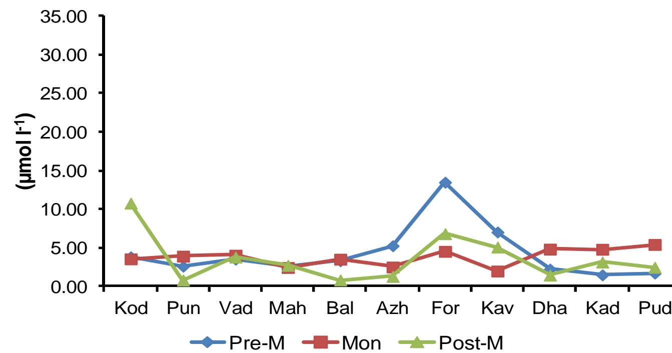


Fig.7. Nitrate: spatial and seasonal variations

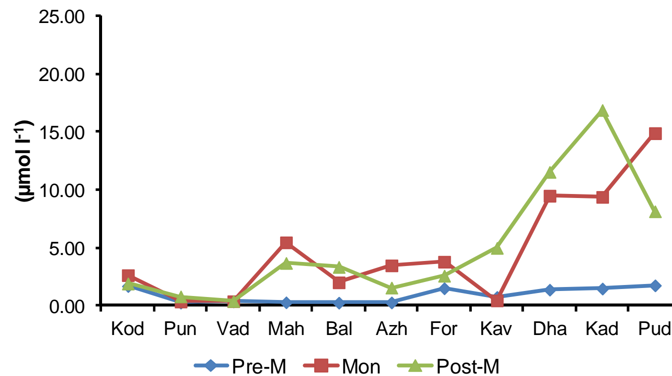
2.3.1.7. Phosphate

In 2006-07, the maximum phosphate concentration (16.88±1.49 µmolL-1) was observed at station 10 during post-monsoon, while the minimum (0.26±0.05 µmolL-1) was observed at station 5 during pre monsoon (Fig.8a). Similarly in 2007-08, the maximum phosphate concentration (19.08±1.69 µmolL-1) was observed at

station 9 during post-monsoon. However, the minimum concentration was observed at stations 2 (0.09±0.57 µmolL-1), 5 (0.09±0.07 µmolL-1) and 1 (0.09±0.60 µmolL-1) during pre monsoon, monsoon and post monsoon seasons, respectively (Fig.8b).

According to two-way ANOVA, PO_4 concentration significantly varied with stations ($F_{10,66}$ = 106.929, $p<0.001$) and with seasons ($F_{2,66}$ = 459.814, $p<0.001$). The results from the independent two-sample (Student's) *t*-test showed that the phosphate concentration was not significantly different between the two samplings ($p>0.05$).

(a) 2006-07



(b) 2007-08

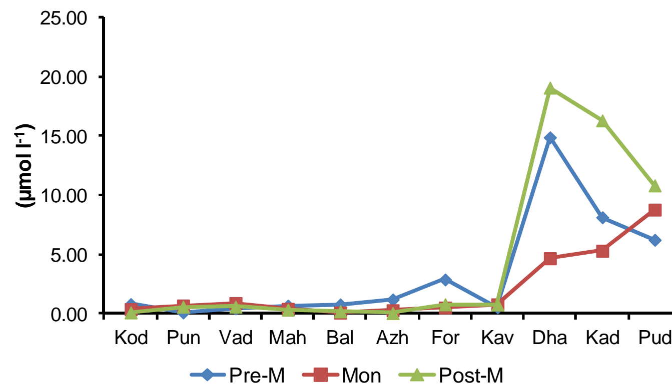


Fig.8. Phosphate: spatial and seasonal variations

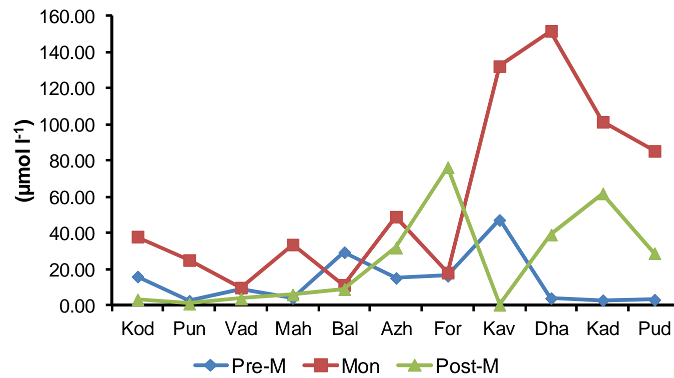
2.3.1.8. Silicate

In 2006-07, the maximum silicate concentration (151.65±8.24 µmolL-1) was observed at station 9 during monsoon, while the minimum (0.38±0.08 µmolL-1) was

observed at station 8 during post-monsoon (Fig.9a). In 2007-08, the maximum silicate concentration (118.5±67.09 µmolL-1) was observed at station 5 during post monsoon, while the minimum (1.11±0.13 µmolL-1) was observed at station 4 during pre monsoon (Fig.9b).

According to two-way ANOVA, SiO_2 concentration significantly varied with stations ($F_{10,66} = 106.929$, $p<0.001$) and with seasons ($F_{2,66} = 459.814$, $p<0.001$). The results from the independent two-sample (Student's) *t*-test showed that the silicate concentration was not significantly different between the two samplings ($p>0.05$).

(a) 2006-07



(b) 2007-08

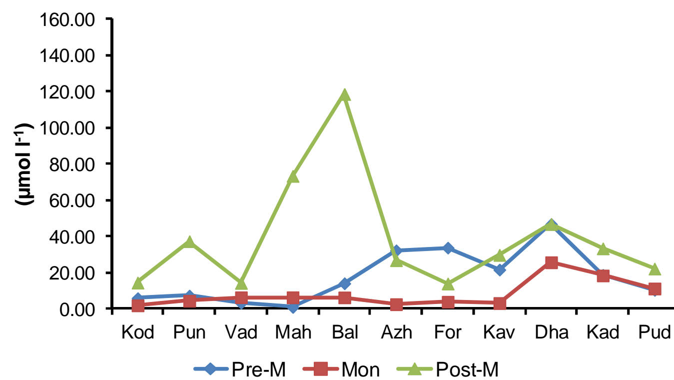


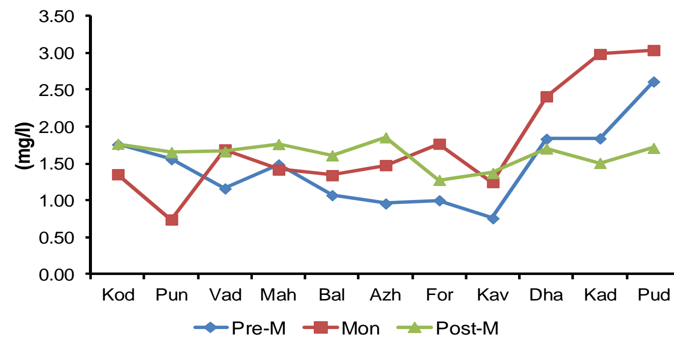
Fig.9. Silicate: spatial and seasonal variations

2.3.1.9. BOD

In 2006-07, the maximum BOD (3.04±0.32 µmolL-1) was observed at station 11 during monsoon and the minimum (0.74±0.05 µmolL-1) was observed at station 2 during the same season (Fig.10a). In 2007-08, the maximum BOD (2.51±0.16 µmolL-1) was observed at station 9 during monsoon and the minimum (0.47±0.26 µmolL-1) was observed at station 1 during the pre monsoon season (Fig.10b).

According to two-way ANOVA, BOD significantly varied with stations ($F_{10,66}$ = 106.929, $p<0.001$) and with seasons ($F_{2,66}$ = 459.814, $p<0.001$). The results from the independent two-sample (Student's) *t*-test showed that the BOD was not significantly different between the two samplings ($p>0.05$).

(a) 2006-07



(b) 2007-08

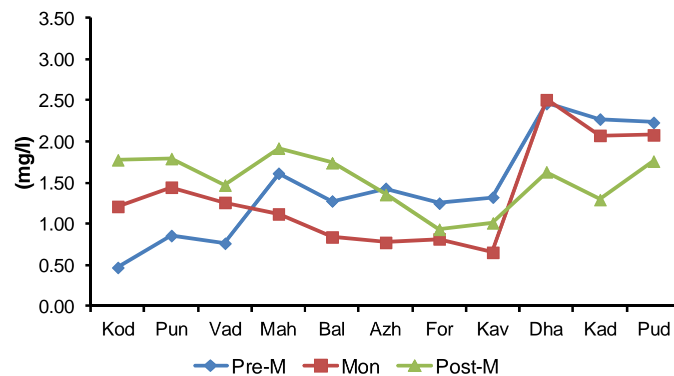


Fig.10. BOD: spatial and seasonal variations

2.3.2. Sediment characteristics

2.3.2.1. Sediment Temperature

The sediment temperature ranged from 23.71±0.23°C (station 9, monsoon) to 30.13±0.21°C (station 11, pre-monsoon) during 2006-07 and from 23.22±0.26°C (station 2, monsoon) to 30.27±0.47°C (station 9, pre monsoon) during 2007-08 (Fig.11).

Two-way ANOVA indicated that sediment temperature significantly varied with stations ($F_{10,66} = 11.401$, $p<0.001$) and with seasons ($F_{2,66} = 285.935$, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the sediment temperature was not significantly different between the two samplings ($p>0.05$).

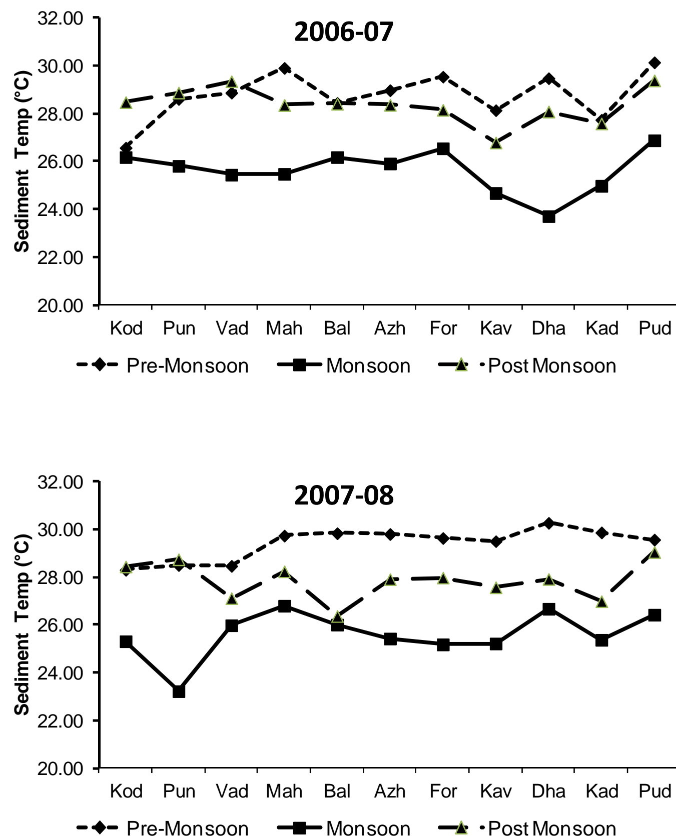


Fig.11. Sediment temperature: spatial and seasonal variations

2.3.2.2. Sediment Ph

The sediment pH ranged from 6.30±0.11 (station 1, monsoon) to 7.70±0.04 (station 11, post-monsoon) during 2006-07 and from 6.65±0.12 (station 1, monsoon) to 8.51±0.13 (station 9, post monsoon) during 2007-08 (Fig.12).

Two-way ANOVA indicated that sediment pH significantly varied with stations ($F_{10,66}$ = 6.035, $p<0.001$) and with seasons ($F_{2,66}$ = 313.739, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the sediment pH was not significantly different between the two samplings ($p>0.05$).

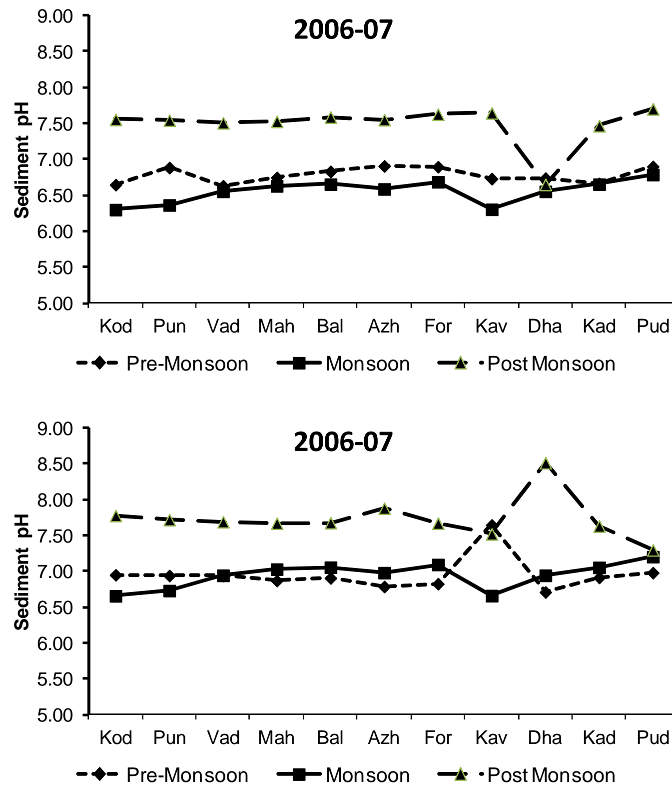


Fig.12. Sediment pH: spatial and seasonal variations

2.3.2.3. Sediment Texture

The percentage composition of the sediment fractions (sand, silt and clay) during Sampling I (2006-07) and Sampling II (2007-08) are presented in Fig.13. The textural composition of the sediments consisted of varying compositions of sand, silt and clay in the different stations during both the sampling.

In 2006-07, the maximum sand concentration (92.93±1.86 was observed at station 5 during monsoon, while the minimum (3.48±0.73 %) was observed at station 3 during the pre-monsoon season. Similarly, the maximum silt concentration (73.14±0.95 %) was observed at station 2 during monsoon and the minimum (2.55±1.08 %) was also observed during the same season at station 5. The maximum clay concentration (93.27±2.96 %) was observed at station 3 during pre-monsoon and the minimum (4.52±0.78 %) was observed at station 5 during the monsoon season.

In 2007-08, the maximum sand concentration (94.05±4.58 %) was observed at station 6 during post monsoon and the minimum (3.03±1.76 %) was observed at station 3 during the same season. The maximum silt concentration (81.40±0.83 %) was observed at station 3 during monsoon, while the minimum (3.58±3.23 %) was observed at station 6 during the post monsoon season. The maximum clay concentration (75.9±2.65 %) was observed at station 11 during pre-monsoon, while the minimum (2.36±1.77 %) was observed at station 6 during the post monsoon season.

According to the two-way ANOVA results, the grain size properties of the sediments showed significant differences between the stations (F_{sand}=304.516; F_{silt}=161.748; F_{clay}=75.875; $P<0.001$) and between the seasons (F_{sand}=367.185; F_{silt}=690.604; F_{clay}=939.396; $P<0.001$). The independent two-sample (Student's) *t*-test indicated significant difference in the grain-size composition between the two samplings ($p<0.05$).

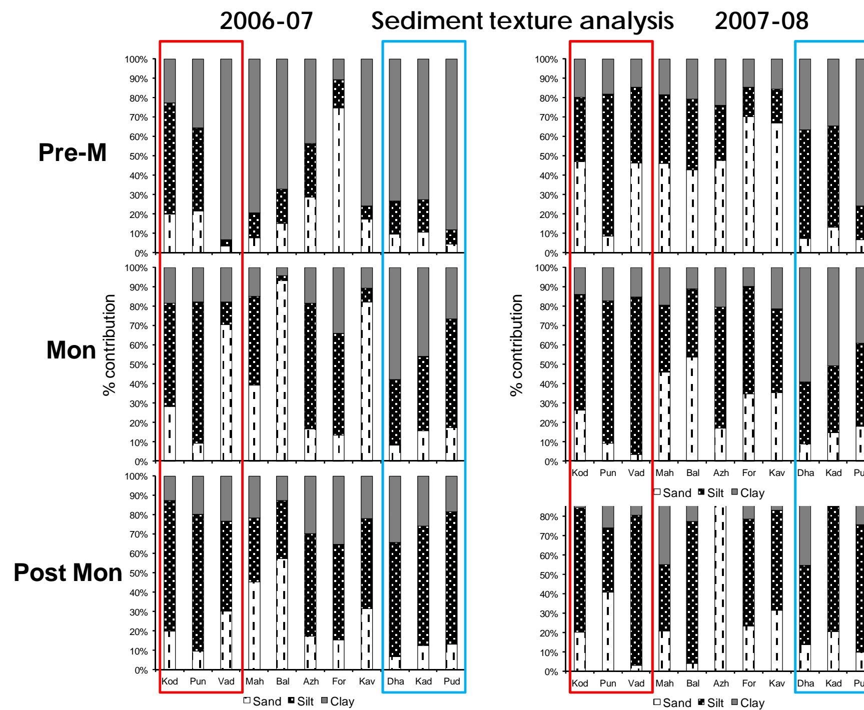


Fig.13. Sediment textural characteristics: spatial and seasonal variations

2.3.2.4. Elemental composition of sediments

The elemental composition of sediments significantly varied between the stations and the seasons. The Carbon content ranged from 0.693 % (station 9, post-monsoon) to 5.792 % (station 1, monsoon) during 2006-07 and from 0.963 % (station 3) to 7.313 % (station 5) in the post-monsoon season during 2007-08. The Nitrogen content ranged from 0.02 % (station 4) to 0.568 % (station 9) in the pre-monsoon season during 2006-07 and from 0.027 % (station 8, monsoon) to 0.518 % (station 5, post-monsoon) during 2007-08. The Sulphur content ranged from 0.052 % (station 2) to 2.346 % (station 10) in the monsoon season during 2006-07 and from 0.105 % (station 1, post-monsoon) to 2.698 % (station 10, pre-monsoon) during 2007-08 (Fig.14).

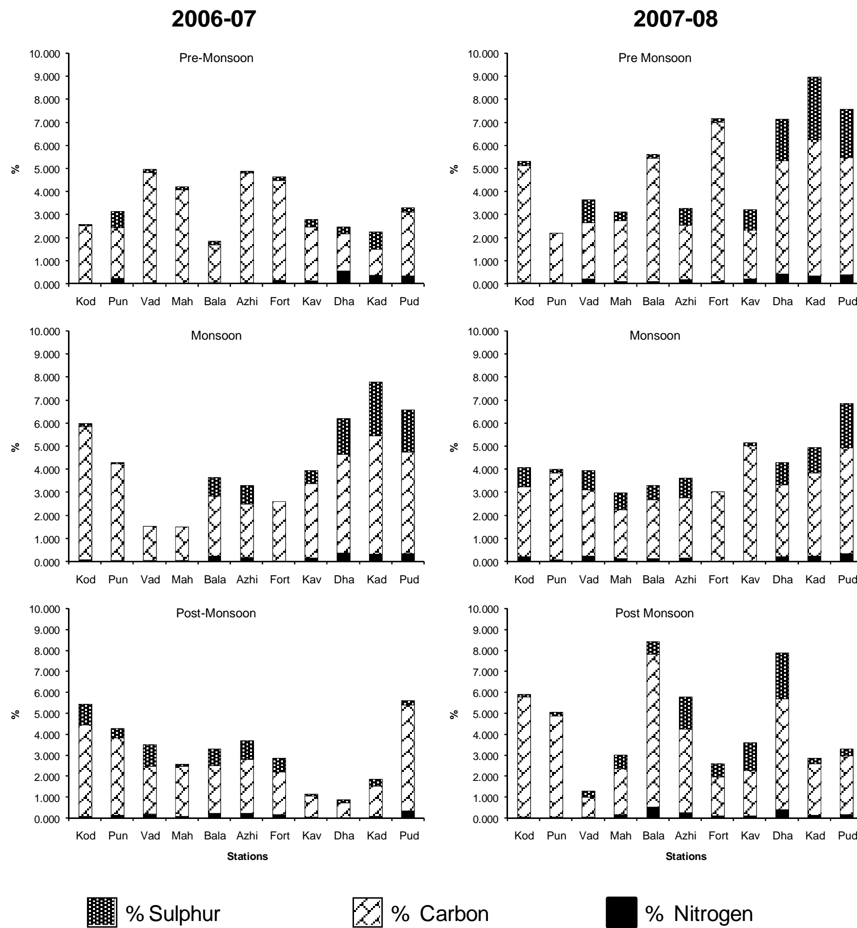
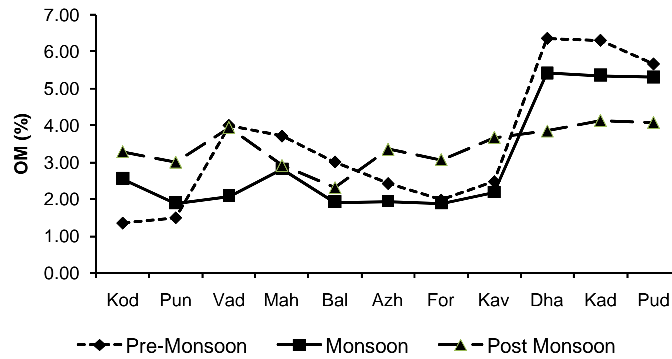


Fig.14. Elemental composition: spatial and seasonal variations

2.3.2.5. Organic Matter

The organic matter (OM) content was relatively high for the mangrove stations in all the three seasons during both the sampling. In 2006-07, the maximum OM content (6.36±0.84%) was observed at station 9 during pre-monsoon, while in 2007-08, the maximum (5.98±0.43 %) was observed at station 11 during monsoon season. Likewise, a minimum OM content of 1.36±0.06% and 1.60±0.03 % was observed at station 1 during the pre-monsoon season in 2006-07 and 2007-08, respectively (Fig.15).

2006-07



2007-08

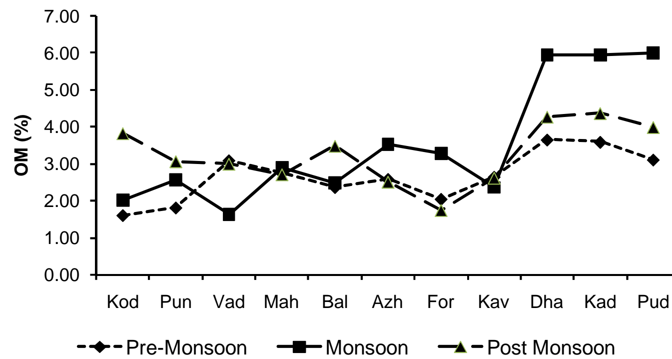


Fig.15. Organic Matter: spatial and seasonal variations

According to two-way ANOVA, OM significantly varied with stations ($F_{10,66}$ = 138.014, $p<0.001$) and with seasons ($F_{2,66}$ = 22.387, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the OM was not significantly different between the two samplings ($p>0.05$).

2.3.2.6. Labile Organic Matter (LOM)

The composition of the labile organic fractions (lipid, protein and carbohydrate) during Sampling I (2006-07) and Sampling II (2007-08) are presented in Fig.16. The labile organic fraction of the sediments consisted of varying compositions of lipid, protein and carbohydrate in the different stations during both the sampling.

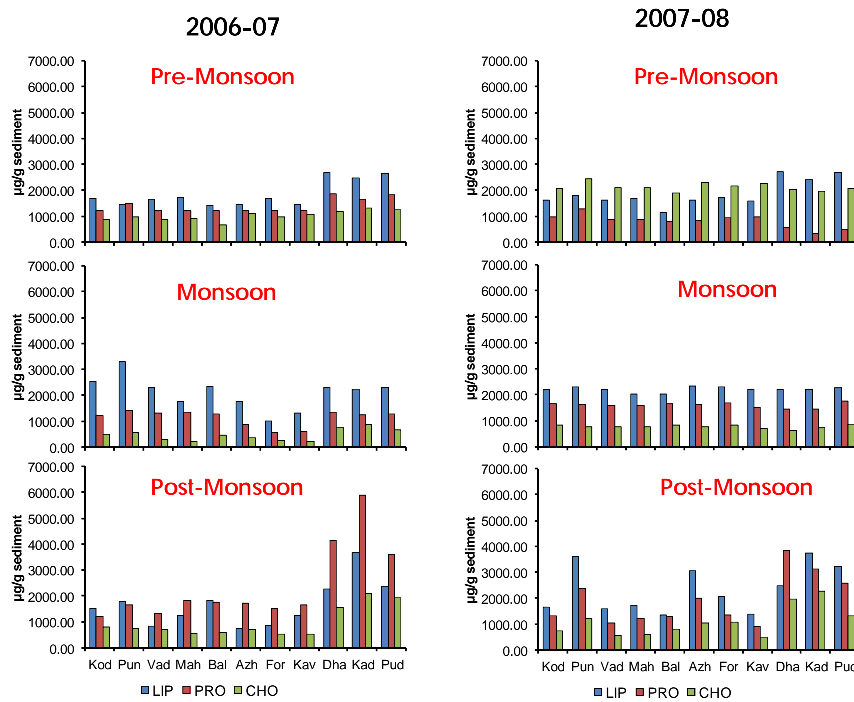


Fig.16. Labile Organic Matter: spatial and seasonal variations

In 2006-07, the maximum lipid concentration (3685.8±130.78 µg/g) was observed at station 10 during post monsoon and the minimum (737.81±57.17 µg/g) was observed at station 6 during the same season. Similarly, the maximum protein concentration (5891.9±537.57 µg/g) was observed at station 10 during post monsoon and the minimum (120.21±0.51 µg/g) was observed during the pre monsoon season at station 6. The maximum carbohydrate concentration (2088.3 ± 32 µg/g) was also observed at station 10 during post-monsoon and the minimum (106.14 ± 56.10 µg/g) was observed at station 8 during the pre-monsoon season.

In 2007-08, the maximum lipid concentration (3735.2 ± 266.3 µg/g) was observed at station 10 during post monsoon, while the minimum (1136 ± 153.51 µg/g) was observed at station 5 during the pre-monsoon season. Similarly, the maximum protein concentration (3858.7 ± 439.7 µg/g) was observed at station 9 during post monsoon and the minimum (325.18 ± 12.72 µg/g) was observed at station 10 during the pre monsoon season. The maximum carbohydrate concentration (2309.2 ± 73.97 µg/g) was also observed at station 6 during pre-monsoon and the

minimum (482.94 ± 54.07 µg/g) was observed at station 8 during the post-monsoon season.

According to two-way ANOVA, the labile organic fraction of the sediments showed significant differences between the stations (Flipid=160.092; Fprotein=12.71; Fcarbohydrate=5.12; p<0.001) and between the seasons (Flipid=103.509; Fprotein=12.71; Fcarbohydrate=5.12; p<0.001). The independent two-sample (Student's) *t*-test indicated significant difference in the LOM concentration between the two samplings (p<0.05).

2.3.2.7. PN (Protein Nitrogen)

The PN content of the sediment ranged from 87.77±16.96 µg/g (station 7, monsoon) to 942.70±86.01 µg/g (station 10, post-monsoon) during 2006-07 and from 52.03±2.04 (station 10, pre monsoon) to 617.39± 70.35 (station 9, post monsoon) during 2007-08 (Fig.17).

Two-way ANOVA indicated that PN significantly varied with stations ($F_{10,66}$ = 106.929, p<0.001) and with seasons ($F_{2,66}$ = 459.814, p<0.001). The result from the independent two-sample (Student's) *t*-test showed that the protein nitrogen content was not significantly different between the two samplings (p>0.05).

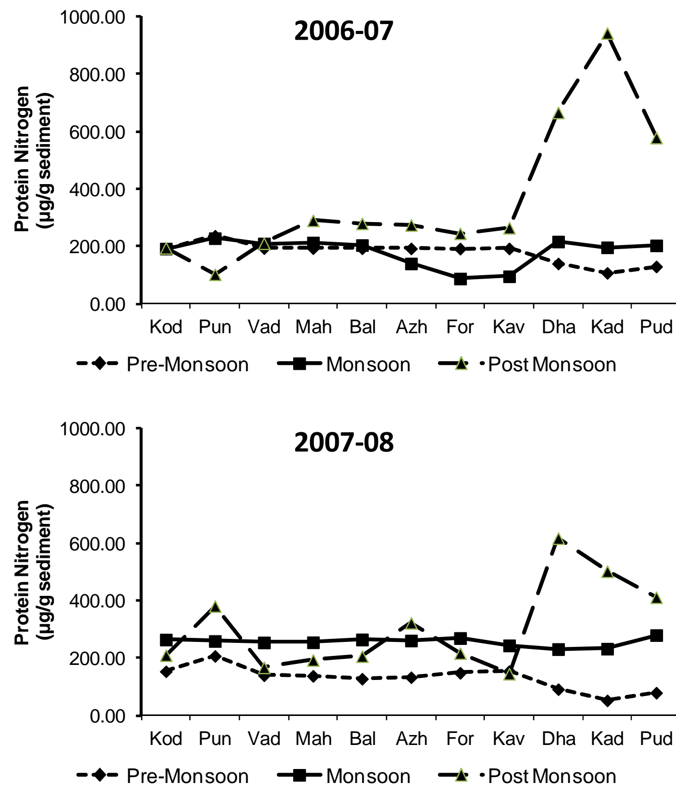


Fig.17. Protein Nitrogen: spatial and seasonal variations

2.3.2.8. Biopolymeric Carbon

The Biopolymeric Carbon content in the sediment ranged from 1119.8±62.76 µg/g (station 7, monsoon) to 6486.7±168.42 (station 10, post-monsoon) during 2006-07 and from 15.39±8.88 (station 10, pre-monsoon) to 5244.5±551.53 (station 10, post-monsoon) during 2007-08 (Fig.18).

Two-way ANOVA indicated that the Biopolymeric Carbon significantly varied with stations ($F_{10,66} = 106.929$, $p<0.001$) and with seasons ($F_{2,66} = 459.814$, $p<0.001$). The result from the independent two-sample (Student's) *t*-test showed that the Biopolymeric Carbon was not significantly different between the two samplings ($p>0.05$).

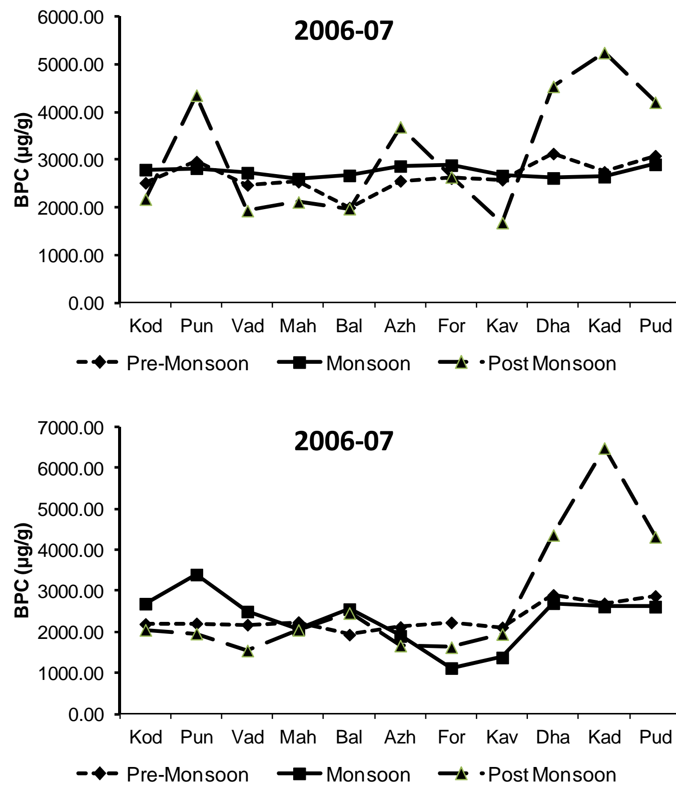


Fig.18. Biopolymeric carbon: spatial and seasonal variations

2.4. Discussion

The tropical coastal zones are highly relevant in the global oceanic scenario, considering their significance in natural and anthropogenic climate change and for oceanic circulation of heat, salt, and water vapour. Although only one-quarter of the world's continental margins lie within the tropics (Jahnke, 2010), most of the world's water and sediment is discharged from tropical rivers (Milliman and Farnsworth, 2011). Highly variable salinities, strong tidal fronts, complex coastal boundary layers, high salinity plugs, tidal trapping and time lags in estuarine circulation, low mean tidal amplitudes, high rates of precipitation and evaporation, permanently stratified thermoclines and haloclines, and migrating fluid mudbanks are some of the environmental characteristics peculiar to tropical coastal waters that have important consequences for biogeochemical cycling (Nittrouer *et al.,* 1995, Alongi *et al.*, 2014).

There is a considerable lack of information on the river deltas along much of the Indian coast and the contribution of these habitats to river-estuary-ocean biogeochemistry. The Arabian Sea is a tropical oceanic zone situated in the north-western Indian Ocean with highly significant biogeochemical fluxes. The water-column processes including the seasonally reversing monsoon-driven circulation and the associated upwelling and productivity/oxygen depletion have been the foci of many previous studies concerning the Arabian Sea from the south-west coast of India. The present study focuses on the enzyme-potential of the benthic microbiota along the major coastal realms, i.e. coastal plains, estuaries and mangroves, of Kerala situated in the south-west coast of India.

Hydrography of near-bottom waters and sediment characteristics are highly significant factors in the biogeochemical cycling in the coastal ecosystems. They in turn determine the diversity and abundance of micro as well as macrobiota. The hydrography as well as sediment characteristics showed significant differences between the stations, as well as between the seasons during the current study. In general, salinity of the near-bottom waters was relatively higher in the coastal stations compared to the estuarine and mangrove stations. This can be attributed to the higher oceanic influence in these stations, while riverine influence is more predominant in the estuarine and mangrove sations. The nutrient parameters did not show any particular trend between the stations.

BOD was relatively high in the mangrove stations, which is well in agreement with the high organic matter (OM) observed in these stations. Mangroves are highly productive ecosystems providing vital ecological and economic resources such as food and fuel, habitats for a variety of fauna including fish, mammals, and invertebrates, protection from waves and currents and most importantly it acts as a net sink for coastal carbon and other elements. Mangroves are among the most carbon-rich biomes (Hogarth, 2007; Nagelkerken *et al.*, 2008; Alongi, 2009; Duarte *et al.*, 2010; Alongi *et al.*, 2014). Mangrove coastal habitats account for 14% of carbon sequestration by the global coastal ocean, referred to as 'blue carbon' (Alongi, 2014). However, mangrove habitats are declining and have a global area of

only < 140,000 km^2 (Alongi *et al.*, 2012). The replanting and restoration of these coastal habitats would help recoup anthropogenic CO_2 emissions and thereby, prevent habitat loss.

The sediment textural analysis indicated that irrespective of seasonal changes, sand, silt and clay dominated the estuarine, coastal and mangrove stations, respectively. The predominance of clay in the mangrove stations can be attributed to the relatively high OM content in these stations. Organic matter in the sediment depends on the texture of the sediments and higher organic matter content is associated with finer fractions of sediments than coarser ones. According to Ramamurthy *et al.,* (1979), the smallest sediment size fraction has more organic material than the largest size. This is because the clayey sediments which offer larger surface area for the adsorption of organic matter would accumulate more organic carbon (Rajamanickam and Setty, 1973). Therefore, clay and clayey-fractions have relatively higher organic matter content than the coarser sediments (De Haas, 2002).

The organic matter content in sediment is a function of several factors like productivity of the region, time of burial, rates of sedimentation and *in situ* biological and chemical activities. The biochemical composition (labile organic matter) of the sediment can be attributed to the enzyme degradation potential of the sediment microbiota. During the current study, lipids dominated the labile organic matter composition, followed by proteins and carbohydrates, the only exception being pre-monsoon in 2007-08, wherein carbohydrates formed the dominant component. Sedimentary lipid concentration has been reported to be the most labile fraction of sedimentary organics and it is considered as a good index in terms of sediment energetics and nutritive quality (Fabiano and Pusceddu, 1998). The sedimentary lipids and proteins were highest in mangrove sediments during the pre and post monsoon seasons, while the coastal sediments had a relatively higher concentration during the monsoon, probably owing to the heavy land runoff. The Biopolymeric carbon, mainly composed of protein and lipid carbon (Fabiano and Danovaro, 1999), was also found to be high in the mangrove sediments. The high lipid content in the sediments can be attributed to the high abundance of diatoms in the Arabian Sea, which are important carriers of lipids to the sediment upon sinking (Budge and

Parrish, 1998; Ramos *et al.,* 2003). Along with diatoms, the sediment microbiota can also be attributed to the high nutritive quality of the sediments (Orejas *et al.,* 2003). Rapid sedimentation promotes the burial of fresh, highly reactive organic matter, while in areas of slow sedimentation; much of the organic matter decomposes (Schuffert *et al.,* 1994). Mangrove ecosystems facilitate the accumulation of fine particles, fostering rapid rates of sediment accretion (Alongi *et al.*, 2014). This explains the high nutritive content of mangrove sediments as compared to coastal and estuarine systems. The higher nutritive value of mangrove sediments could possibly be one of the reasons for its selection as a breeding ground by many marine fauna, including fishes. Further studies on the microbial community and their potential for degradation of organic matter would help to determine the role of microbiota in enhancing the nutritive value of sediment biome as well as biogeochemical cycling of vital elements.

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CHAPTER 3

OCCURRENCE AND DISTRIBUTION OF TOTAL HETEROTROPHIC BACTERIA IN THE COASTAL SEDIMENTS

3.1. Introduction

Aquatic environment and marine habitat in particular is an ideal habitat for the growth and survival of microorganisms. Microorganisms by and large from all of the taxonomic groups are represented in aquatic habitats including the sediment substratum. A sharp separation between sediment bacteria and aquatic bacteria is not possible as the inland and nearshore waters are constantly exposed to the flora of the soil origin. Studies on distribution pattern of heterotrophic bacteria in the seas were initiated by Zobell (1946) and followed by Kriss (1963) and Sieburth (1971). Within sediments, there is often a heterogeneous mixture of particles of different size, origin and surface features. These alterations influence the number and composition of the microbial community (Romani and Sabater, 2001).

Estuaries, mangroves and the nearshore sediments are considered as a productive zone for diverse population of microorganisms. The microbial communities found in the marine and brackish water environments play a significant role in the decomposition of organic matter and mineralization (Rheinheimer, 1980). Quantification of bacterial numbers and biomass is important to the understanding of their ecological role in many environments. Identifying the primary factors responsible for their regulation is a major goal of microbial ecology, hence the need for an assessment of methods. Heterotrophic bacteria and their processes in the environment give an understanding on their abundance, distribution, production and their involvement in nutrient cycling and how they are contributing towards the microbial food web. Microorganisms inhabiting in the marine ecosystems participate in an important role of decomposing a wide spectrum of organic compounds ranging in molecular size from monomers to polymers and thereby its biogeochemical cycling (Chrost, 1991). Sinking particles mediate the transfer of carbon to the deep-sea and thus transport of energy.

The specific microbes associated with sedimentation of the sinking particles in the ocean's interior remain largely uncharacterized (Fontanez *et al.*, 2015). Although bacterial communities have been frequently studied, recent studies show that there is a need to characterize the marine environment with respect to its microflora (Gontang *et al.*, 2007; Wilson *et al.,* 1986). Even though great deal of research has been achieved on the biogeography of marine microorganisms many unknown facts still remain which needs to be elucidated for the better understanding of their complexity (Joseph *et al.*, 2003). It was generally noticed that microbial populations are more abundant in muddy sediments than in sandy ones depending on the granulometry of particles (Lakshmanaperumalsamy *et al.*, 1986). Quantification of bacterial numbers and biomass is important to our understanding of the ecological role of bacteria in any environment. Identifying the primary factors responsible for the regulation of bacterial numbers is a major goal of microbial ecology, hence the need for an assessment of methods.

The bacterial population depends on changes in water temperature, salinity, abundance of organic nutrients, and on other physico-chemical parameters (Azam *et al.*, 1983; *Fong et al.*, 1993). However, it has been recognized that bacterial population may be considerably modified by interactions with biotic factors (Martin and Bianchi, 1980). Though a cosmopolitan distribution of free-living bacteria has been proposed to be the governing rule behind prokaryotic life, the knowledge on the distribution of marine sediment bacteria is still in its infancy (Finlay, 2002).

In general, the marine microbial studies of Arabian Sea were concentrated chiefly on the water column and reports on the microbiological aspects from benthic environment are scarce. Bacterial heterotrophy has been extensively measured over most oceanic regions (Ducklow and Carlson, 1992). Heterotrophic bacteria present in the water and sediment of marine and brackish water environments and their role in the decomposition of organic matter and regeneration of minerals have been investigated by Azam and Hodson (1977) and Pomeroy (1979). The role of microbial communities in the process of decomposition of organic matter, nutrient regeneration from sediments, biogeochemical cycling and production of particulate matter in marine environment is recognized (Rheinheimer, 1980). However, studies on the

seasonal distribution of various bacterial taxa (in the marine and estuarine environments are limited (Lakshmanaperumalsamy *et al.*, 1986). During the past years, very few studies have been conducted on the quantitative distribution of heterotrophic bacteria in Indian coastal sediments (Dhevendran, 1977). Hence, an attempt has been made to quantify the THB in the coastal, estuarine and mangrove systems along the Kerala coast.

3.2. Materials and Methods

3.2.1. Estimation of the abundance of Heterotrophic Bacteria

Sediment samples were collected using a Van Veen Grab ($0.025m^2$). Sediment sub-samples for isolation of heterotrophic bacteria were collected using a sterile spatula and were aseptically transferred into sterile and labelled polypropylene bags (Fig. 3.1). The samples were immediately stored at 4-5°C till analysis with 25% v/v of glycerol in filtered and autoclaved seawater. The samples were analyzed after suitable dilutions with sterile sea water (10^{-4} and 10^{-5}) and plating onto ZoBell's 2216e Marine Agar medium by spread plate method (Rheinheimer, 1997). The agar medium was sterilized by autoclaving at 121°C for 15 minutes. The sterilized medium was poured into sterile petri dishes. After surface drying, the inoculation was done using a sterile glass spreader. The plates were incubated at 28 ± 2°C for 5-7 days in a bacteriological incubator. The bacterial colonies developed after the incubation period were counted and expressed as colony forming units (cfu) per gram dry weight of sediment)

Fig. 3.1. Sediment collection for isolation of total Heterotrophic Bacteria

3.2.2. Isolation and Preservation of Cultures

The cultures after incubation for the specified time were counted for determining the cfu (Fig. 3.2). The colonies were counted and distinctive colonies were numbered and streaked on to nutrient agar plates. The pure cultures thus obtained were transferred onto nutrient agar slants. For preservation, the pure cultures were streaked on to nutrient agar slants in glass vials and layered with sterile mineral oil and was preserved indefinitely.

Fig. 3.2. Distinct bacterial colonies obtained by spread plate method

3.2.3 Enumeration of total bacteria using Epifluorescence microscopy

Bacteria have been enumerated in several ways, with many of the same techniques being applied in diverse systems. Increasingly, direct counts are used in studies of bacterial population densities rather than traditional spread plate methods, which have been shown to significantly underestimate numbers (ZoBell, 1946).

To estimate the total count, one gram of the pooled sediment sample in hexamine buffered formalin (2%) was dispersed in sterile saline and sonicated in a water bath for 10 minutes to dislodge the cells. Enumeration was done following the method of Hobbie *et al.*, (1977). Preserved samples (1 ml) were stained (in duplicate) with 100 µl of acridine orange stain (final concentration 0.01% w/v) and incubated in dark for 2 minutes and then filtered through 0.22 µm black stained Nuclepore polycarbonate membrane filter. The slide was then viewed under oil immersion objective (100x) of

an epifluorescence microscope (Olympus Corporation, Japan), equipped with HbO lamp and U-MWB2 mirror unit having excitation filter of 460–490 nm and emission filter of 520 nm. The average field count (calculated from the field views) was used to calculate the total bacterial abundance as [**Filter area / Area of counted field x Average count x 1 / Volume of sample x 1/ Dilution**]. The bacterial abundance was expressed as cells g^{-1}.

3.2.4. Statistical Analysis

The data were analysed by univariate and multivariate statistical methods. Two-way ANOVA (SPSS v. 16) was employed to determine the spatial and temporal variations in the THB density. Cluster analysis using Bray-curtis similarity indices (PRIMER v.6) was employed to determine the resemblance profile of the heterotrophic bacterial population between the various stations. To select the variables that best explain the distribution of hetertrophic bacteria in the sediment, the BIOENV analysis was carried out (Spearman rank correlation method) as integrated in PRIMER v.6. (Clarke and Ainsworth, 1993).

3.3. Results

3.3.1. Distribution of cultivable heterotrophic bacteria

Total (culturable) heterotrophic bacteria (THB) in the surface sediments of the study area exhibited significant spatio-temporal variations.

3.3.1.1. Spatio-temporal variations in THB density in 2006-07

During 2006-07, of all the coastal, estuarine and mangrove stations, the lowest THB density was observed at the mangrove station Dharmadam (Stn. 9) during monsoon ($3.63 \pm 1.62 \times 10^5$ cfu g^{-1}) and the highest was observed at the coastal station Vadi ($134.99 \pm 6.99 \times 10^5$ cfu g^{-1}) during the same season. In general, the coastal stations had the highest THB count, followed by estuarine stations and the lowest count was observed at the mangrove stations during all the three seasons.

During the pre monsoon season, the highest THB density was observed at Vadi (92.98 ± 5.36 x 10^5 cfu g^{-1}) and the lowest at Dharmadam (6.63 ± 0.64 x 10^5 cfu g^{-1}). Likewise, in the monsoon season also, the maximum THB density was observed at station Vadi (134.99 ± 6.99 x 10^5 cfu g^{-1}), while the minimum was observed at station Dharmadam (3.63 ± 1.62 x 10^5 cfu g^{-1}). During the post monsoon season also, the maximum THB density was observed at Punnapra (97.50 ± 4.86 x 10^5 cfu g^{-1}) while the minimum was recorded at Kadalundi (3.83 ± 0.75 x 10^5 cfu g^{-1}; Fig. 3.3).

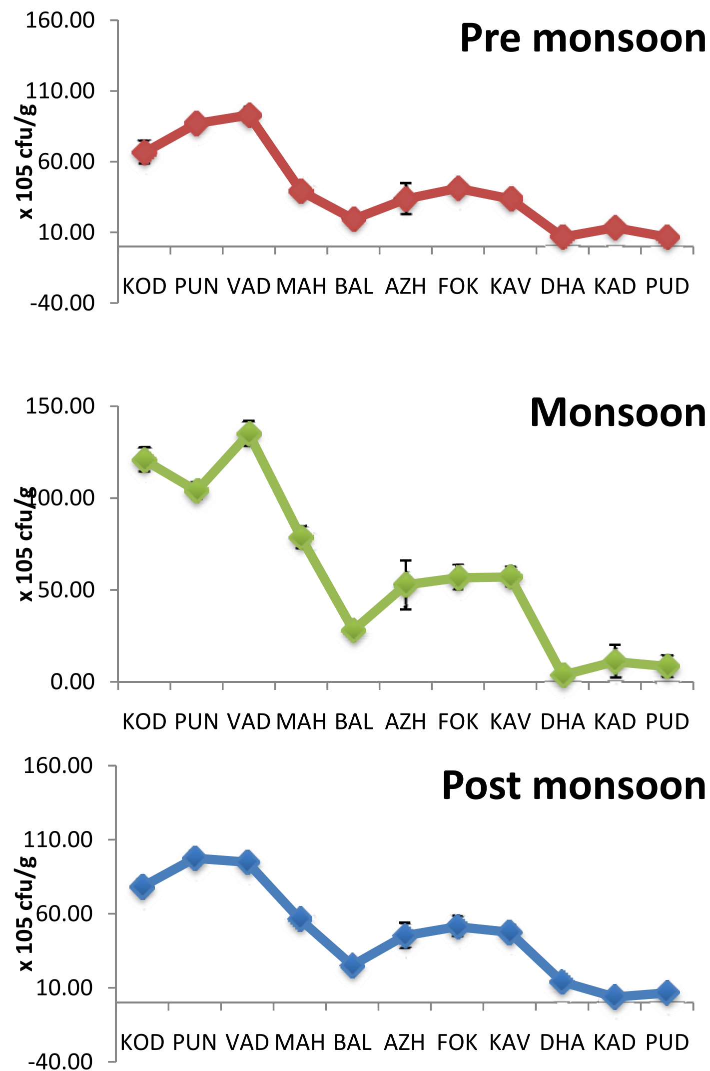

Fig.3.3. Total Heterotrophic Bacterial population during various seasons along the study area in 2006-07

3.3.1.2. Spatio-temporal variations in THB density in 2007-08

During the 2007-08 sampling, the lowest THB density was observed at Punnapra during post monsoon ($0.94 \pm 0.26 \times 10^5$ cfu g^{-1}) and the highest was observed at Mahe ($150.28 \pm 5.71 \times 10^5$ cfu g^{-1}) during the monsoon season. Even though the lowest THB density was observed in the mangrove stations (the only exception being the coastal station 2, Punnapra, during the post monsoon season) as in the 2006-07 sampling, there was a slight change in the THB population trend in the pre monsoon and monsoon seasons, compared to 2006-07, with the estuarine stations having relatively higher THB count than the coastal stations.

During the pre monsoon season, the highest THB density was observed at Fort Kochi ($66.52 \pm 2.26 \times 10^5$ cfu g^{-1}) and the lowest at Puduvaipu ($7.67 \pm 2.07 \times 10^5$ cfu g^{-1}). Likewise, in the monsoon season also, the maximum THB density was observed at Mahe ($150.28 \pm 5.71 \times 10^5$ cfu g^{-1}), and the minimum was observed at Dharmadam ($4.84 \pm 0.79 \times 10^5$ cfu g^{-1}). During the post monsoon season also, the maximum THB density was observed at Kavanad ($55.73 \pm 1.71 \times 10^5$ cfu g^{-1}). However, the minimum THB density was observed at Punnapra ($0.94 \pm 0.26 \times 10^5$ cfu g^{-1}), followed by Puduvaipu ($5.31 \pm 0.52 \times 10^5$ cfu g^{-1}; Fig. 3.4).

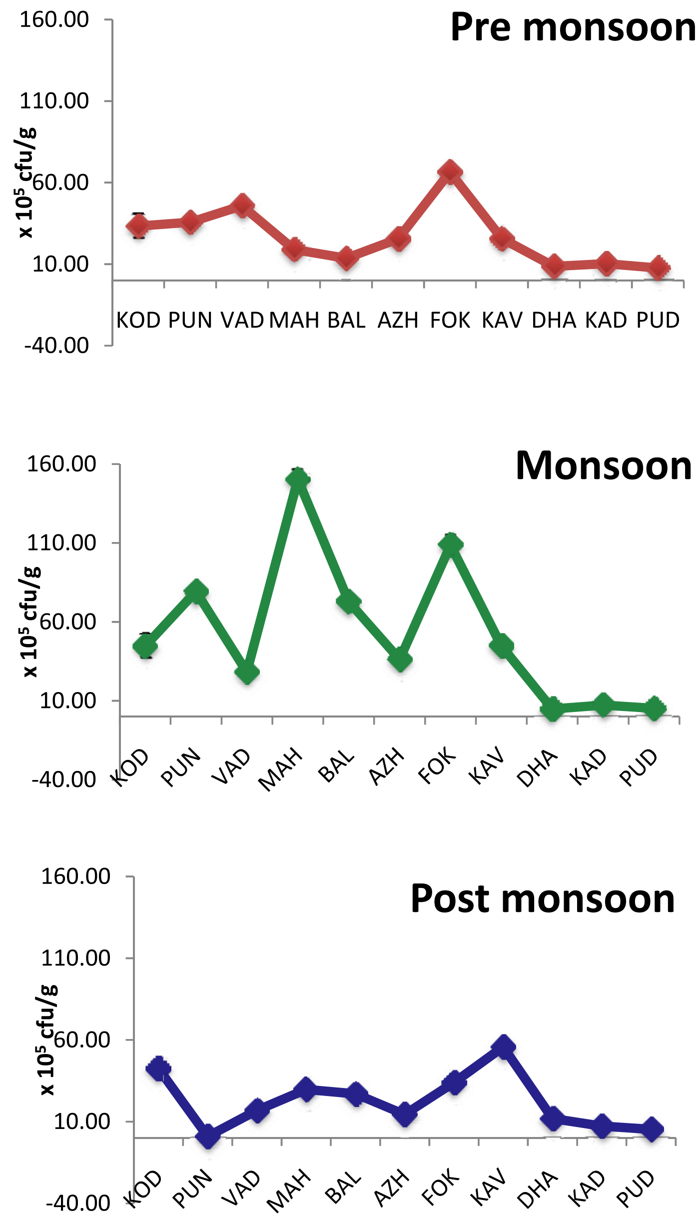

Fig.3.4. Total Heterotrophic Bacterial population during various seasons along the study area in 2007-08

3.3.2. Bacterial density in Total Direct Count (TDC)

The TDC varied from 11.13 x 10^8 cfu /g at Mahe to 22.9 x 10^8 cfu /g at Kodikkal. In general, the coastal stations had the highest TDC, followed by mangrove stations, while the TDC was relatively lower in the estuarine stations (Fig. 3.5).

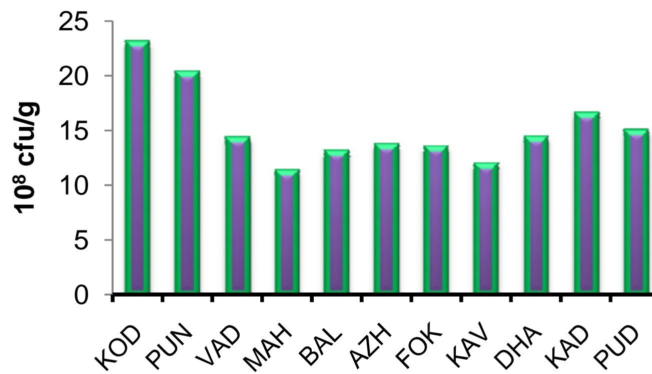

Fig.3.5. Total Direct Count of the heterotrophic bacterial population in the various stations along the study area

3.3.3. Statistical Analysis

According to two-way ANOVA, THB significantly varied between stations ($F_{10,66} = 346.312$, $p<0.001$) and between seasons ($F_{2,66} = 96.399$, $p<0.001$).

Cluster analysis

The similarity profile using Bray Curtis similarity index indicated the formation of three distinct clusters by the coastal (stations 1-3), estuarine (stations 4-8) and mangrove (9-11) stations during 2006-07 (Fig. 3.6). The only exception was the clustering of the estuarine station 4, Fort Kochi along with the coastal stations. During 2007-08 also, the mangrove stations formed a distinct cluster. However, there was slight intermixing of coastal and estuarine stations.

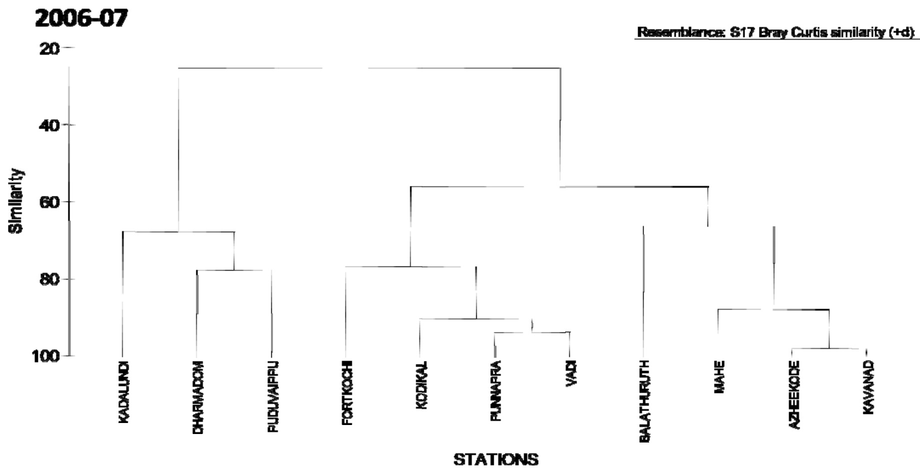

Fig. 3.6. Similarity profile of THB between stations

BIOENV Analysis

The interrelationships between measured environmental variables and heterotrophic bacteria were investigated by BIOENV analysis using PRIMER software and the Pearson correlation coefficient matrix.

Table 3.1. BIOENV analysis: The combination of environmental variables generating the highest rank correlations between similarity matrices of population and environmental data

SEASON	HIGHEST RANK CORRELATION	VARIABLES
PRM	0.774	pH Nitrate Lipid Protein Biopolymeric carbon Protein Nitrogen
MON	0.903	Salinity
POM	0.925	Salinity pH Dissolved Oxygen Nitrite Nitrate Phosphate Silicate Biopolymeric carbon Organic Matter

BIOENV analyzed the correlation between Bray-Curtis similarity matrices for environmental variables and THB population. These correlations are repeated for all possible combinations of the measured environmental variables and the set of environmental variables that produce the highest correlation are those that most accurately explain the distribution of heterotrophic bacteria in the sediments.

During pre monsoon, the highest rank correlation with THB data (R=0.774) was obtained with pH, Nitrate, Lipid, Protein, Biopolymeric carbon and Protein Nitrogen. During the monsoon, only two variables, salinity and chlorophyll *a*, contributed to the highest rank correlation (R=0.903), while during post monsoon, the highest rank correlation (R=0.925) was obtained with a set of nine variables, namely, Salinity, pH, Dissolved Oxygen, Nitrite, Nitrate, Phosphate, Silicate,

Biopolymeric carbon and Organic Matter. The results indicate that the THB population density depends on a set of key environmental parameters.

3.4. Discussion

The abundance and distribution of total heterotrophic bacteria have a direct bearing on other forms of nutrients in different compartments of the environment. Several studies have already been carried out to characterize heterotrophic bacteria in ocean sites and in different coastal areas of temperate, tropical and polar zones (Billen, 1990; Ducklow *et al.,* 1993; Hopkinson *et al.*, 2002). The benthic microbial environment is dominated by a complex widespread network of bacterial populations (Du *et al.*, 2011). The bacteria in the sediment-water interface are of great biogeochemical and ecological importance considering their role in decomposition, mineralisation and subsequent recycling of organic matter (Walsh, 1991). While earlier works on marine microbial studies were concentrated on the water column, chiefly during JGOFS (Joint Global Ocean Flux Studies; 1992-97), work on the microbiological aspects from benthic environment of Arabian Sea are relatively less.

The current study assessed the THB of three distinct ecosystems, coastal, estuarine and mangrove ecosystems, along the Kerala coast. The observed THB density of $3.63 \pm 1.62 \times 10^5$ to $134.99 \pm 6.99 \times 10^5$ cfu g^{-1} during 2006-07 and $0.94 \pm 0.26 \times 10^5$ to $150.28 \pm 5.71 \times 10^5$ cfu g^{-1} during 2007-08 of the current study is comparable to earlier reports. In a study carried out along the 50 m to 150 m of the continental shelf region from Mangalore to Tuticorin, the THB in sediment varied from 13×10^5 to 610×10^5 cfu/g sediment (Pujari *et al*., 2004). Likewise, in another study from the central Adriatic Sea, bacterial counts ranged between $(1.5 \pm 0.2) \times 10^8$ cells g^{-1} and $(53.1 \pm 16.0) \times 10^8$ cells g^{-1} in sandy and muddy sediments, respectively (Luna *et al.,* 2002). Such incidence of relatively high THB in nearshore waters can be attributed to the anthropogenic activities such as sewage discharge, agricultural land runoff, industrial effluent discharge etc.

Among the coastal ecosystems, mangrove ecosystems have been reported to be rich in bacterial flora (Lakshmipriya and Sivakumar, 2012). In tropical

mangroves, bacteria and fungi have been attributed to 91% of the total microbial biomass (Alongi, 1988). According to Lakshmipriya and Sivakumar (2012), the THB population along various sites in Pitchavaram varied from 2.84×10^8 to 1.86×10^9 cfu/g. Similarly, THB counts of mangrove litter in Vellar estuary has been reported to range from 0.24×10^8 to 0.95×10^8 cfu/g (Kathiresan *et al.*, 2011). However, during the current study, the lowest THB density was observed in the mangrove stations during all the three seasons. The observed trend in THB density was coastal > estuary > mangrove. According to BIOENV results, pH, Nitrate, Lipid, Protein, Biopolymeric carbon and Protein Nitrogen are the key factors driving the THB population during pre monsoon season. Among these, pH and nitrate did not exhibit any particular trend between the stations. However, lipid and BPC was relatively high and protein and PN was relatively low in the mangrove stations compared to the other stations. Das *et al.,* (2011) have reported that the Labile Organic Matter (LOM) determines the total bacterial counts. Therefore, the observed decrease in protein and PN, in spite of high lipid content, might have caused a decrease in THB density.

During monsoon, salinity was identified as the major decisive factors of THB density. Incidentally, both these parameters registered the lowest values in the mangrove stations compared to coastal and estuarine stations. Among the nine parameters exhibiting highest rank correlation to THB during post monsoon also, salinity, along with DO was lowest in the mangrove stations, while phosphate, BPC and OM recorded relatively high values. Bacterial population has been reported to be highly correlated to salinity (Painchaud and Therriault, 1989), which explains the decrease in THB density in the low saline mangrove stations, in spite of a high BPC and OM content. However, the observed correlation between THB and chlorophyll *a* could be due to the dependency of chlorophyll *a* on salinity (Painchaud and Therriault, 1989). The DO concentration of the interstitial water is also positively correlated to the sediment microbial population. Therefore, the low DO concentration during post monsoon season in the mangrove stations can also be attributed to the relatively low occurrence of THB in these stations.

Acosta *et al*., (2006) observed that seawater temperature is the principal determinant of seasonal distribution of culturable heterotrophic bacteria in mangrove

sediments. However, during the current study, temperature did not exhibit any particular trend between the stations, and therefore, cannot be attributed to the observed variations in THB density. Over and above all the attributed key parameters, the constant anthropogenic influence probably causing frequent population shifts could be the major reason behind the observed relatively low THB density in the mangrove stations along Kerala coast during all the three seasons.

In case of marine samples, colony-forming units (cfu) obtained by traditional plate culture techniques do not mimic the real environmental conditions under which natural populations flourish (Ward *et al.,* 1990). Besides, it has been pointed out that only 1% of the bacteria present in natural seawater can be cultured and isolated on standard laboratory media as they occur in a state of dormancy or under starvation or are mostly inactive (Bianchi and Giuliano, 1996). Therefore, culturing organisms remains an important step in the process of understanding the biology and ecology of microbial species (Connon and Giovannoni, 2002). The observed three-fold increase in heterotrophic bacterial population (10^8 cells/g as compared to 10^5 cfu/g by count culture method) in the various stations along the study area by the Total Direct Count (TDC) method during the current study testifies this view.

Another interesting observation is the relative increase in bacterial density in the mangrove stations during enumeration using the TDC method. This indicates that the observed low THB in the mangrove stations could possibly be a virtual scenario, while in reality the mangrove ecosystem may be rich in uncultivable bacterial population. The delicate symbioses in which microbes exist in the mangrove environment could be the reason behind them remaining uncultured. Many of the microbes in the mangrove ecosystem could be dependent on syntrophs for supply or removal of a particular nutrient or product, thereby facilitating growth (Nealson, 1997).

The THB profile of the three distinct near-shore ecosystems along the Kerala coast, thus indicates rich population density, except for the relatively low population density in the mangrove stations. The observed deficiency in THB density in the mangrove stations has been mainly attributed to the prevalent low salinity and low

DOES conditions in these stations. Moreover, enumeration of bacterial population by TDC exhibited considerable increase in cell number indicating towards the uncultivable nature of majority of the population in this ecosystem. Further studies on the generic composition of the sediment bacterial population would help in better understanding of this intricate microbiome.

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CHAPTER 4

GENERIC DISTRIBUTION OF TOTAL HETEROTROPHIC BACTERIA ALONG THE COASTAL SEDIMENTS

4.1. Introduction

A microbial community and its genomic diversity associated with a particular ecosystem or habitat forms a microbiome (Fierer *et al.*, 2012; Ruff *et al.*, 2015). The fundamental aspect concerning the study of microbiomes is the identification of assembly rules that govern microbial community structure and community function (Nemergut *et al.,* 2013). Communities of heterotrophic bacteria have been reported to be the driving force behind oxidation of deposited organic matter, regeneration of inorganic nutrients, and trophic food-web system (Fenchel *et al.,* 1998).

Eventhough several studies have been undertaken to derive the nature and functions of bacterial biomes, there still remains a lot of lacunae, especially with regard to sediment bacterial biomes. Research on cultivated bacterial isolates and the process of biogeochemical cycles have provided limited information about the role of microbes in the sediments (Capone and Kiene, 1988). The benthic microbial communities are very distinct from pelagic communities owing to the changes incurred by water depth, sediment depth and energy availability in the form of deposited organic matter (Boer *et al.,* 2009; Zinger *et al.,* 2011; Ruff *et al.,* 2015). These microbial communities promote organic matter degradation and decomposition in sediments and overlying water, releasing dissolved organic and inorganic substances, thereby supporting the main biogeochemical cycles of the benthic ecosystem like primary production and remineralization of organic material (Decho, 2000; Hewson *et al.,* 2007; Raulf *et al.,* 2015). The sediment bacterial action thus reflects the hydrological structure and nutrient levels in the marine environment. Fluctuations in key microbial taxa have been attributed to the dynamics of important biogeochemical processes (McCalley *et al.,* 2014). However, the mechanisms that underlie the participation of microbiomes in marine food webs and biogeochemical cycles are not clearly deciphered. The biochemical interactions of microorganisms

with ocean systems at the nanometer to millimeter scale, which is relevant to microbial activities, needs to be analysed at the grass-root level (Oppenheimer, 1963; Azam and Malfatti, 2007).

Heterotrophic bacterial abundance determines the nutrient availability in different compartments of the environment. In view of this, investigations on the role of heterotrophic bacteria in the decomposition of organic matter and regeneration of minerals have been undertaken (Azam and Hodson, 1977; Pomeroy, 1979). It is presumed that the environmental conditions prevalent in a given environment decide the heterotrophic bacterial population assemblage in the ecosystem. The key factors underlying the bacterial community assembly have been identified as dispersal, ecological drift, environmental selection, and diversification (Hanson *et al.,* 2012). Considering the dynamic nature of coastal ecosystems owing to water, wind, sea waves and human impact (Mudryk *et al.*, 2011), studies on the heterotrophic bacterial population is challenging as well as highly important (Zedler, 2000).

Various researchers have attempted characterisation of heterotrophic bacteria in the coastal sediments of temperate, tropical and polar zones. The determination of the extent of diversity is mainly restricted to culturable bacteria since majority of strains are uncultivable. It is estimated that more than 99% of microscopic organisms cannot be cultivated by routine techniques (Pace, 1997; Stackebrandt, 2001). Cavallo *et al.*, (1999) reported gram positive bacilli as abundant genus, along with *Aeromonas, Photobacterium* and *Pseudomonas* from the coastal sediments of Ionian Sea. Concerning the Indian waters, considerable research has focused on the heterotrophic bacterial community of the Arabian sea and Bay of Bengal shelf sediments. Nair *et al.* (1978) studied the sediment bacterial population and physiological responses of the bacterial isolates from the coastal regions of India. Chandrika and Girijavallabhan (1990) studied the quantitative and qualitative distribution of heterotrophic bacteria from Bay of Bengal in relation to environmental parameters. Ramaiah *et al.* (1996) reported the culturable heterotrophic bacterial numbers, generic composition and uptake of labeled glucose by microbial assemblages in Bay of Bengal. Ramaiah *et al.* (2004) studied the abundance of pollution indicator bacteria from Mumbai coast (Arabian Sea).

Goltekar *et al.* (2006) studied the irretrievability of marine heterotrophic bacteria from coastal to deep regions of Arabian Sea and Bay of Bengal. Recent studies on the distribution and diversity of bacterial diversity along the shelf sediments of Bay of Bengal identified *Bacillus*, *Vibrio* and *Alteromonas* as the dominant genera (Jacob *et al.,* 2013). In another study focusing the Arabian Sea shelf sediments, *Bacillus*, *Alteromonas*, *Vibrio*, Coryneforms and *Micrococcus* were identified as the dominant heterotrophic bacterial groups (Ramya *et al.*, 2013).

The present study is focused on characterizing the culturable heterotrophic bacteria along the Kerala coast, south western Arabian Sea continental shelf region. Evaluation of the variations in heterotrophic bacterial community involved in the decomposition of organic matter from the three distinct ecological realms comprising the study area, i.e., coastal, estuarine and mangrove ecosystems, is attempted.

4.2. Materials and Methods

4.2.1. Isolation and purification of the bacterial strains

The total heterotrophic bacteria (THB) were isolated as described in chapter 3. The strains were purified in nutrient agar plates by conventional streak plate method. The cultures were repeatedly streaked on nutrient agar plates for purity and preserved in sterile nutrient agar vials overlaid with sterile liquid paraffin.

4.2.2. Identification of the bacterial strains

The purified isolates were identified up to respective genera based on cell morphology and biochemical reactions as per Bergey's Manual of Determinative Bacteriology (Holt and Krieg, 1994). Standard bacteriological procedures such as colony morphology, pigmentation, Gram staining and endospore staining were performed to identify the isolates up to generic level following the scheme given by Oliver (1982) and Skerman (1949, 1957, 1959, 1967, 1974) and Skerman et al. 1980. The strains were grouped into different generic groups based on their Gram reaction, spore formation, aerobic growth, cell shape and arrangement, motility, presence of oxidase enzyme and oxidative-fermentative ability. They were further characterized

based on selected tests for each group as per Bergey's Manual of Determinative Bacteriology.

4.2.2.1. Gram staining

The foremost routine test for classifying bacteria is the gram stain, developed in 1884 by Danish scientist Hans Christian Gram (September 13, 1853 – November 14, 1938), a differential stain as it discriminate among two types of the bacteria found and distinguish them based on differences in their cell wall. For staining, smears were prepared on clean glass slides using 12-18 hours old bacterial cultures. The primary stain, ammonium oxalate crystal violet was added to the fixed smear and allowed to stand for one minute. Then the slides were rinsed gently in running water. Then Gram's iodine solution was added as a mordant and allowed to stand for one minute. The slides were washed and treated with acetone (decolourizer) for 30 seconds. After washing the smear, the counter stain safranin-O was added and allowed to stand for one minute. The smear was rinsed, allowed to air dry and observed under oil immersion objective lens of a microscope. The walls of gram positive bacteria have more peptidoglycan than the gram negative bacteria which help to retain the original violet dye and cannot be counterstained. Gram negative bacteria on the other hand have thinner walls, containing an outer layer of lipopolysaccharide, which is dissolved by the alcohol treatment which permits the original dye leaching out and the cell to take up the counter stain. Thus, gram-positive bacteria stain violet, and gram-negative bacteria stain pink.

4.2.2.2. Mannitol Motility Test

Mannitol motility medium was prepared and about 3-4 ml was distributed in test tubes. The tubes were sterilized in an autoclave and left for setting in a vertical position. After cooling, the inoculum from the culture was stabbed straight to the bottom. The tubes were then incubated at room temperature for 48-72 hours. Change of color from pink to yellow in the medium showed the utilization of mannitol. Motile bacteria moved away from the line of inoculation and exhibited diffused growth

4.2.2.3. Spore staining

Gram positive strains were subjected to spore staining. Smears were prepared using 60-72 hours old cultures. The slides were flooded with malachite green and allowed to react at room temperature for one minute. Then the slides were periodically heated by using a Bunsen burner until steam arose from the stain on the slide. The slides were steamed for about 3 minutes, replacing the malachite green as it evaporated from the slides, and were then allowed to cool for about 5 minutes before rinsing with water. Then the counter stain safranin-O was added and allowed to stand for one minute. The slides were then washed and allowed to air dry, and observed under oil immersion objective lens of a microscope. Spores appeared in green colour and the vegetative cells were pink in colour.

4.2.2.4. Oxidase Test

Small pieces of filter paper (Whatman paper No.1) were soaked in 1% aqueous solution of N,N,N',N'-tetramethyl-p-phenylene diamine dihydrochloride and the papers were dried. A small portion of the culture was placed on the test paper with a clean platinum loop. Oxidase positive cultures showed a blue color within 10-30 seconds.

4.2.2.5. Catalase Test

On a clean glass slide, a smear of fresh bacterial culture was prepared. A drop of concentrated hydrogen peroxide (H_2O_2) solution was added on the smear. Effervescence or bubbling was noticed in the case of cultures producing catalase enzyme.

4.2.2.6. Marine Oxidation Fermentation (MOF) Test

MOF medium was prepared and about 5 ml was distributed in test tubes and sterilized in an autoclave. After sterilization, the tubes were kept in a slanting position. The inoculum was stabbed and streaked on the agar slant and incubated for 48 hours. Oxidative forms showed a change in colour from pink to yellow from slope to bottom. Gas production was observed by the presence of cracks and bubbles in the

hard agar of the butt area. Alkaline reactions were noticed by a deep pink color in the slope region.

4.2.2.7. O/129 Test

Detection of resistance or sensitivity to 2.4-iamino-6.7-diisopropylteridine, known as *Vibrio*static Agent O/129, can be useful for differentiating certain bacterial species. This test is carried out using disks impregnated with this substance (discs loaded with 0.5 mg). This test was used to distinguish between *Vibrio* and *Aeromonas*. Discs were prepared using 2, 4-diamino-6, 7-di-isopropylpteridine phosphate. The discs were placed on swabbed nutrient agar plates and incubated overnight at 30° C. Sensitive cultures showed a zone of inhibition around the disc. No zone of inhibition around disk or resistance to *Vibrio*static Agent 0/129 is shown by *Aeromonas, Enterobacteriaceae, Pseudomonas, Alcaligenes, Achromobacter Alteromonas, Flavobacterium* and *Staphylococcus* whereas it is sensitive to the *Vibrio*static agent 0/129 for *Vibrio*, *Flavobacterium* and *Micrococcus*.

4.2.2.8. Antibiotic sensitivity (Kirby-Bauer) Test

Penicillin G sensitivity was tested to distinguish between *Pseudomonas* and *Alteromonas*. It was also used to distinguish between *Moraxella* and *Acinetobacter*. Muller Hinton agar plates were swabbed with 24 hour old bacterial broth and the antibiotic discs were placed and the plates were incubated overnight at 37° C. Clearing zone around the disc indicated sensitivity to the antibiotic.

4.2.2.9. Polymixin B Sensitivity

Sensitivity to antibiotics polymixin B disc, was determined after incubation for 24–48 h at 30°C on Marine Agar for the differentiation of *Flavobacterium* and *Cytophaga*.

4.2.2.10. Indole test

The indole test is used to check the release of indole from the breakdown of tryptophan. Cultures were inoculated into tryptone broth and incubated at 37°C for

48 hrs. Tryptone broth is rich in the aminoacid tryptophan, which can be used by some bacteria as a source of carbon and nitrogen, as well as energy. As the isolates grow in tryptone broth, they attack the tryptophan and degrade it to indole, pyruvic acid and ammonia. After incubation, 1ml of Kovac's reagent was added to the medium, which will interact with indole to form rosindol dye (bright red compound). Bacteria producing red ring at the surface of the medium were considered as indole positive.

4.2.2.11. Arginine Dihydrolase Test

This test was used to distinguish between *Vibrio* and *Aeromonas*. The medium contains bromocresol purple as pH indicator. As the dextrose in the medium is fermented, the medium changes from purple to yellow. If both decarboxylation and deamination occurs alkaline end products are formed.

4.2.2.12. DNase Test

This test was used to distinguish between DNase producing *Alteromonas* from other Gram-negative forms. The DNA agar plates were spot inoculated and incubated at room temperature (28+2 °C) overnight. Flooded the plate with 1N HCl. Clearing around the colonies indicates the DNase activity. The HCl reacts with unchanged deoxyribonucleic acid to give a cloudy precipitate.

4.2.2.13. Voges – Proskauer (VP) Test

Many bacteria ferment carbohydrates with the production of acetyl methyl cabinol or its reduction product 2, 3 butylene glycol. Acetoin and butylene glycol are detected by oxidation to diacetyl at an alkaline pH, and the addition of α- naphthol which forms a red coloured complex with diacetyl. Buffered glucose broth was inoculated with pure culture and incubated at 37°C for 48 hrs. After incubation 1mL of α-naphthol solution and 1mL of KOH solution were added and shaken vigorously. The development of red colour is considered as positive result.

4.2.2.14. Citrate utilization Test

This is a test for the ability of an organism to utilize sodium citrate as the sole carbon and energy source for growth. Simmons citrate agar tubes were inoculated by using a needle to stab the butt and streak the slant. Development of deep Prussian blue colour after incubation at 37°C for 48 hrs indicates a positive result.

4.2.2.15. Urease test

Bacteria, particularly those growing naturally in an environment, may decompose urea by means of the urease enzyme. The occurrence of this enzyme can be tested by growing the organism in the presence of urea and testing for the production of ammonia by means of a suitable pH indicator. Christensen's urea agar slants were used for the test.

4.2.3. Statistical Analysis

The generic data were analysed by univariate and multivariate statistical approach using Plymouth Routines In Multivariate Ecological Research (PRIMER) version 6 (Clarke, 1993; Anderson et al., 2008). Based on generic composition, the similarity among the stations were analysed by hierarchical agglomerative cluster analysis and non metric-multidimensional scaling (MDS) based on Bray-Curtis similarities and the results obtained were plotted as ordination graphs. A K-dominance curve was plotted to establish the relative dominance of bacterial genera during different seasons. The diversity indices, i.e., Shannon-Wiener diversity index, H'; Peilou's evenness index, J'; Margalef's richness, d and Species dominance, D were also analyzed (PRIMER 6).

4.3. Results

From the coastal ecosystems of Kerala, a total of 2380 bacterial cultures were isolated during 2006-07 and 2412 cultures during 2007-08. The isolates were purified and identified using morphological, physiological and biochemical methods upto the generic level.

4.3.1. Variations in generic composition of heterotrophic bacteria

4.3.1.1. Spatio-temporal variation of heterotrophic bacterial generic composition during 2006-07

In the overall percentage dominance of heterotrophic bacteria isolated during 2006-07, *Bacillus* (19.12%) was the most dominant genus, followed by *Vibrio* (17.39%), *Enterobacteriaceae* (7.31%), *Arthrobacter* (7.02%), *Pseudomonas* (6.47%), *Aeromonas* (5.92%), *Alcaligenes* (4.83%), *Achromobacte*r (4.58%), *Micrococcu*s (2.98%), *Brevibacterium* (2.90%), *Aerococcus* (2.77%), *Psychrobacter* (2.48%), *Aerobacter* (2.39%), *Acinetobacter* (2.27%), *Cytophaga* (1.93%), *Corynebacterium* (1.76%), *Staphylococcus* and *Chromobacterium* (1.22% each), *Moraxella* (1.18%), *Flexibacter* (1.13%), *Alteromonas* (1.09%), *Flavobacterium* (0.97%), *Photobacterium* (0.76%) and *Planococcus* (0.29%, Fig. 4.1).

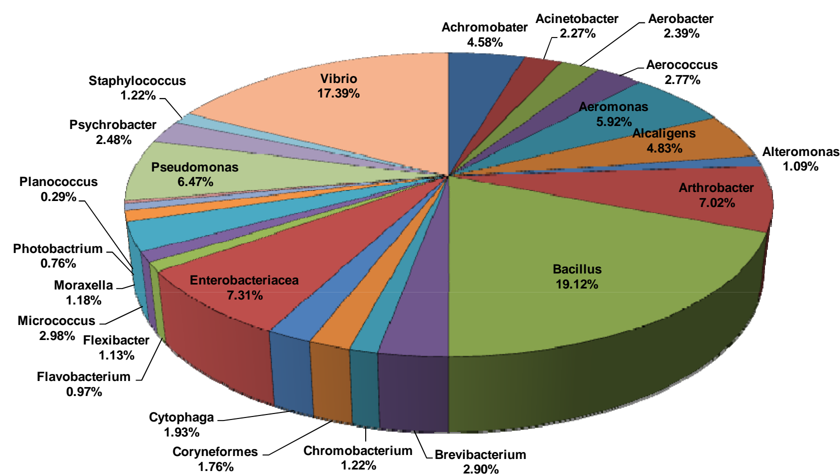


Fig. 4.1. The overall generic composition of culturable heterotrophic bacteria isolated during 2006-07

The coastal stations Kodikkal (Fig.4.2a), Punnapra (Fig.4.2b) and Vadi (Fig.4.2c) had a higher dominance of *Bacillus* during pre monsoon (Kodikkal – 27%; Punnapra – 26%; Vadi – 31%) and post monsoon (Kodikkal – 22%; Punnapra – 21%; Vadi – 25%), and *Vibrio* was found to be most dominant genus during monsoon (Kodikkal – 22%; Punnapra – 11%; Vadi – 20%). Except Fort Kochi (Fig. 4.2g), all the estuarine stations, namely, Mahe (Fig.4.2d), Balathuruth (Fig.4.2e), Azheekode (Fig.4.2f) and Kavanad (Fig.4.2h), had a higher dominance of *Bacillus*

during pre monsoon (Mahe – 34%; Balathuruth – 31%; Azheekode – 33%; Kavanad – 33%) and post monsoon (Mahe – 18%; Balathuruth – 19%; Kavanad – 21%; Azheekode – *Vibrio* was slightly more dominant at 20% compared to *Bacillus* 19%). During monsoon, *Vibrio* was the dominant genus at Mahe (21%), Balathuruth (31%), Azheekode (25%) and Fort Kochi (20%), while *Arthrobacter* dominated Kavanad (16%). *Vibrio* was the dominant genus during pre monsoon also at Fort Kochi (33%), while *Bacillus* dominated the post monsoon season (19%). In the case of mangrove stations, all the three stations, Dharmadom (Fig.4.2i), Kadalundi (Fig.4.2j) and Puduvaipu (Fig.4.2k) had a higher dominance of *Bacillus* during pre monsoon (Dharmadom – 16%; Kadalundi – 26%; Puduvaipu – 38%). However, there were considerable variations in generic dominance between the mangrove stations during monsoon and post monsoon. Among these, Dharmoadom and Kadalundi exhibited some similarity with *Bacillus* being the dominant genus in both these stations during post monsoon (Dharmadom – 20%; Kadalundi – 24%). During monsoon, *Vibrio* was the dominant genus at Dharmadom (15%), while *Vibrio* and Enterobacteriaceae were co-dominant at Kadalundi (19%). The mangrove station Puduvaipu had a relative abundance of *Cytophaga* during both monsoon (24%) and post monsoon (29%).

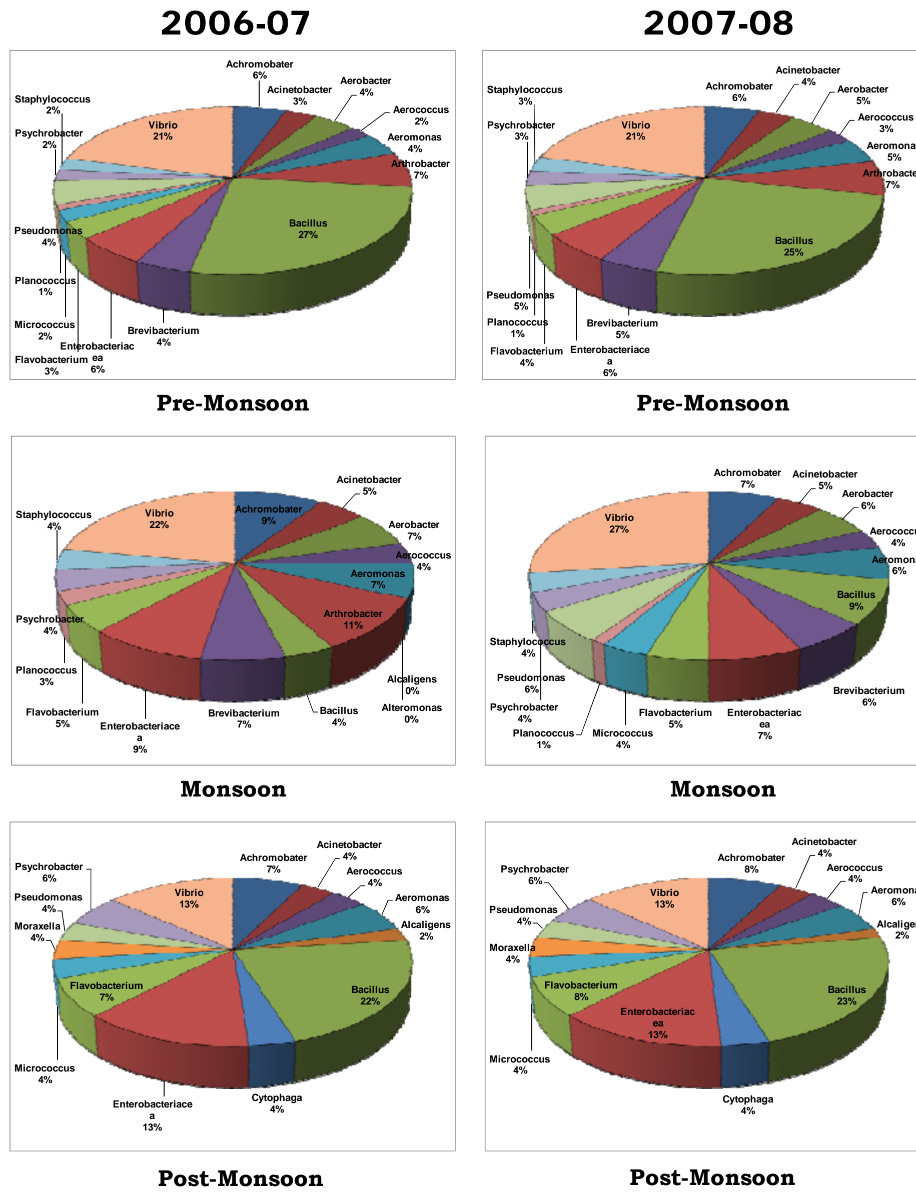


Fig.4.2a. Temporal variations in Heterotrophic bacterial composition at the coastal station Kodikkal during 2006-07 and 2007-08

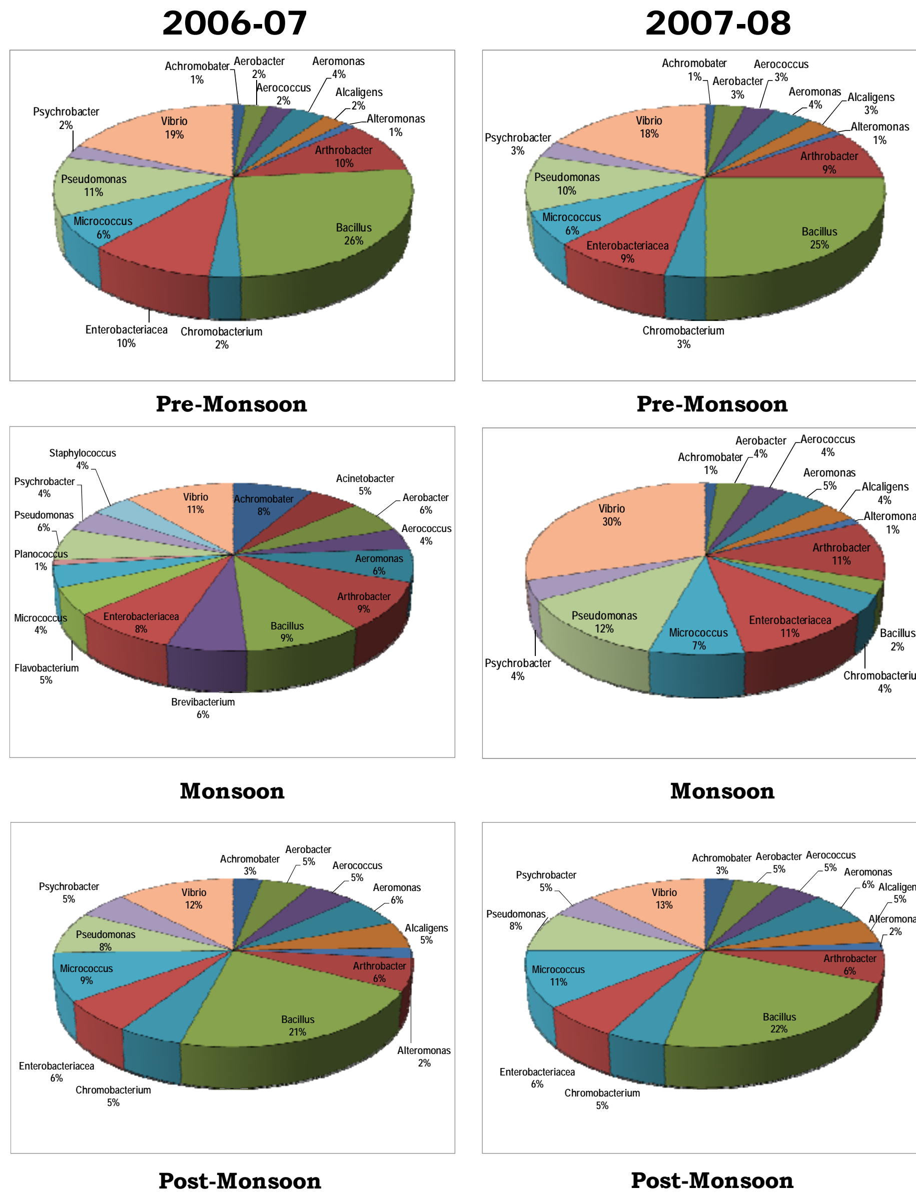


Fig.4.2b. Temporal variations in Heterotrophic bacterial composition at the coastal station Punnapra during 2006-07 and 2007-08

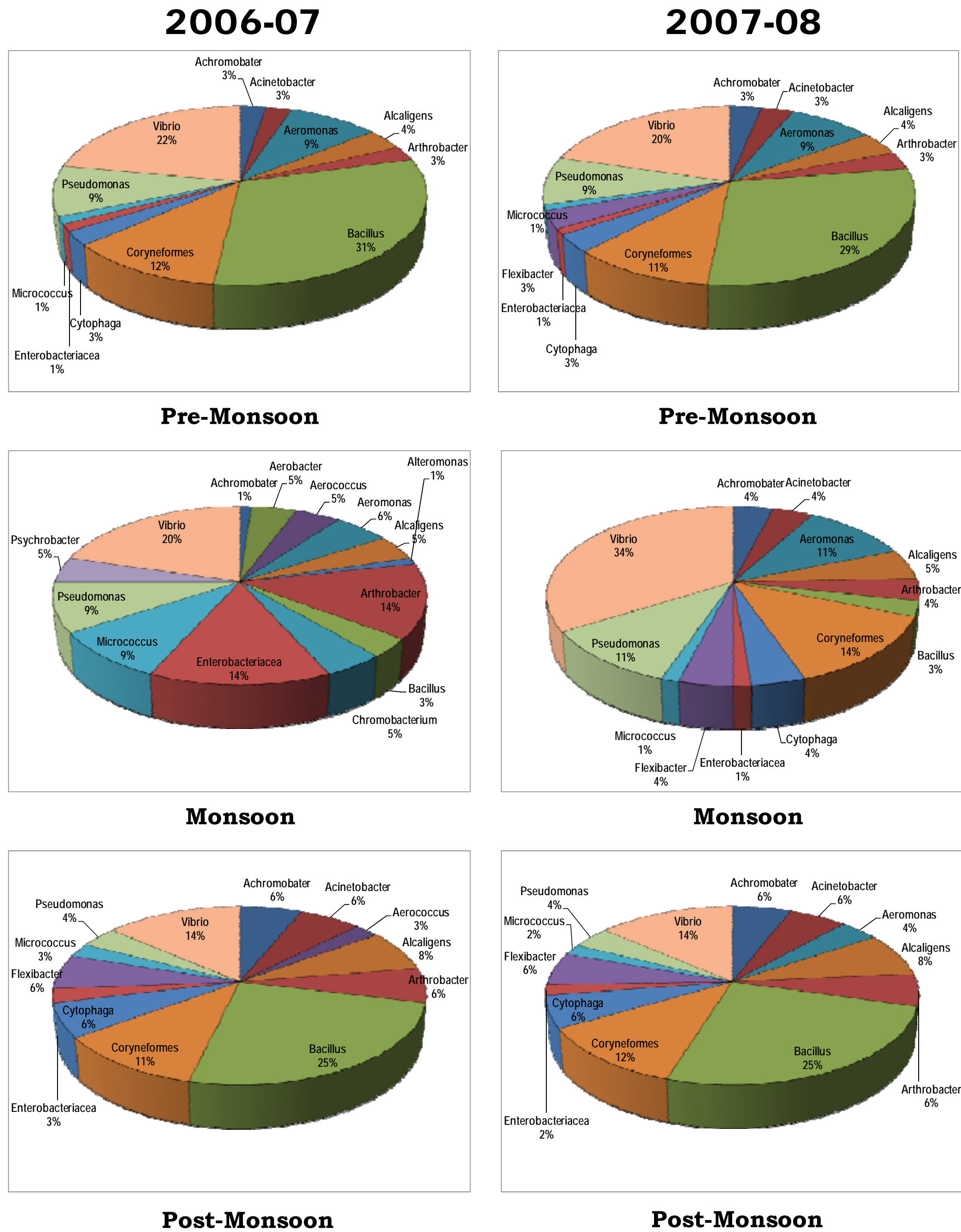


Fig.4.2c. Temporal variations in Heterotrophic bacterial composition at the coastal station Vadi during 2006-07 and 2007-08

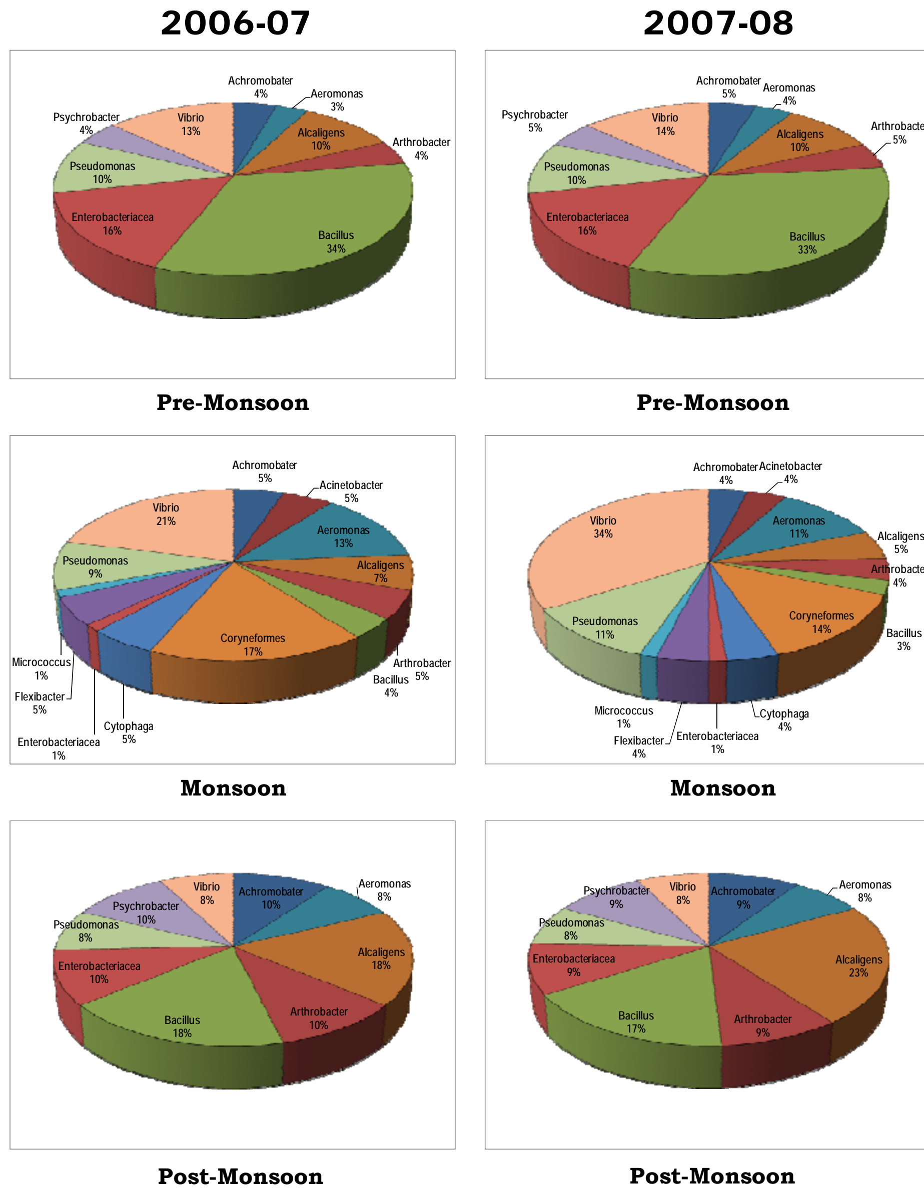


Fig.4.2d. Temporal variations in Heterotrophic bacterial composition at the estuarine station Mahe during 2006-07 and 2007-08

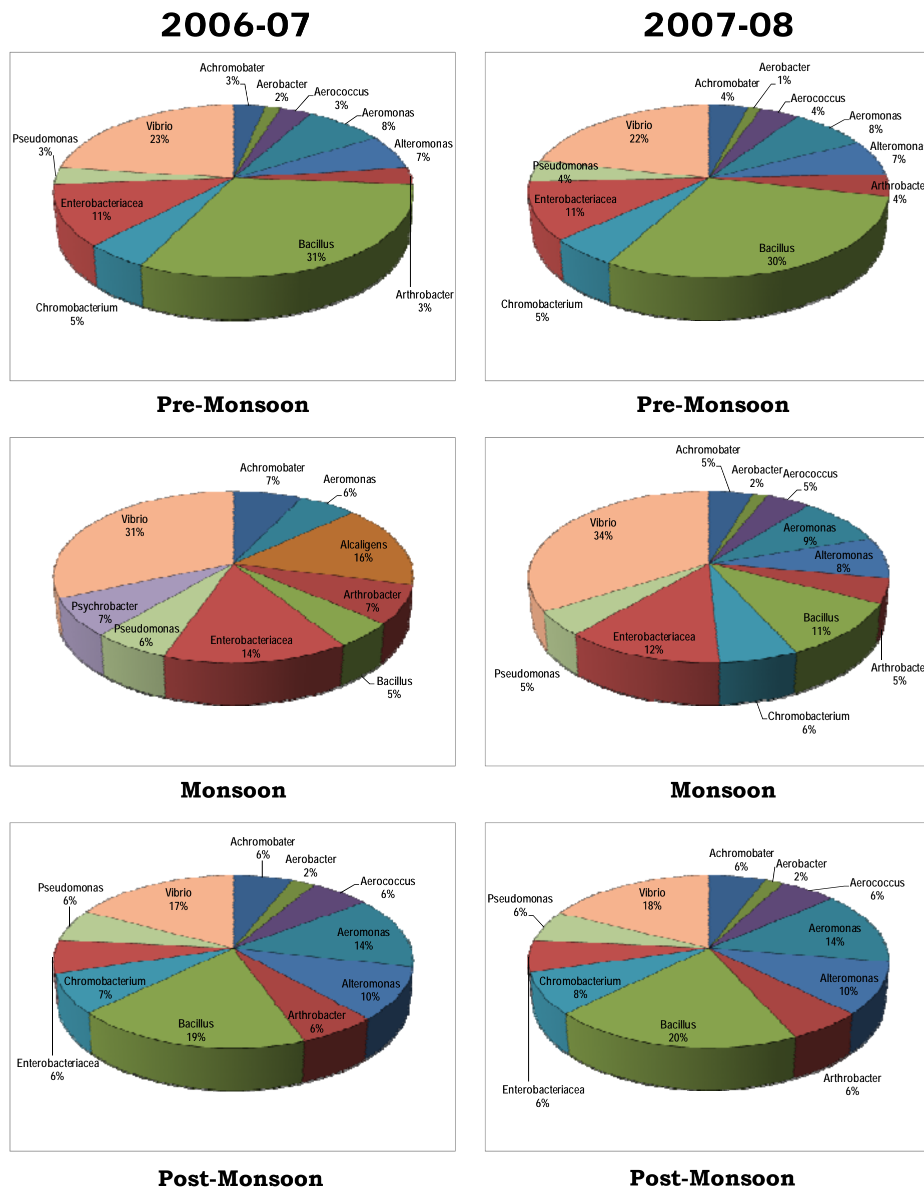


Fig.4.2e. Temporal variations in Heterotrophic bacterial composition at the estuarine station Balathuruth during 2006-07 and 2007-08

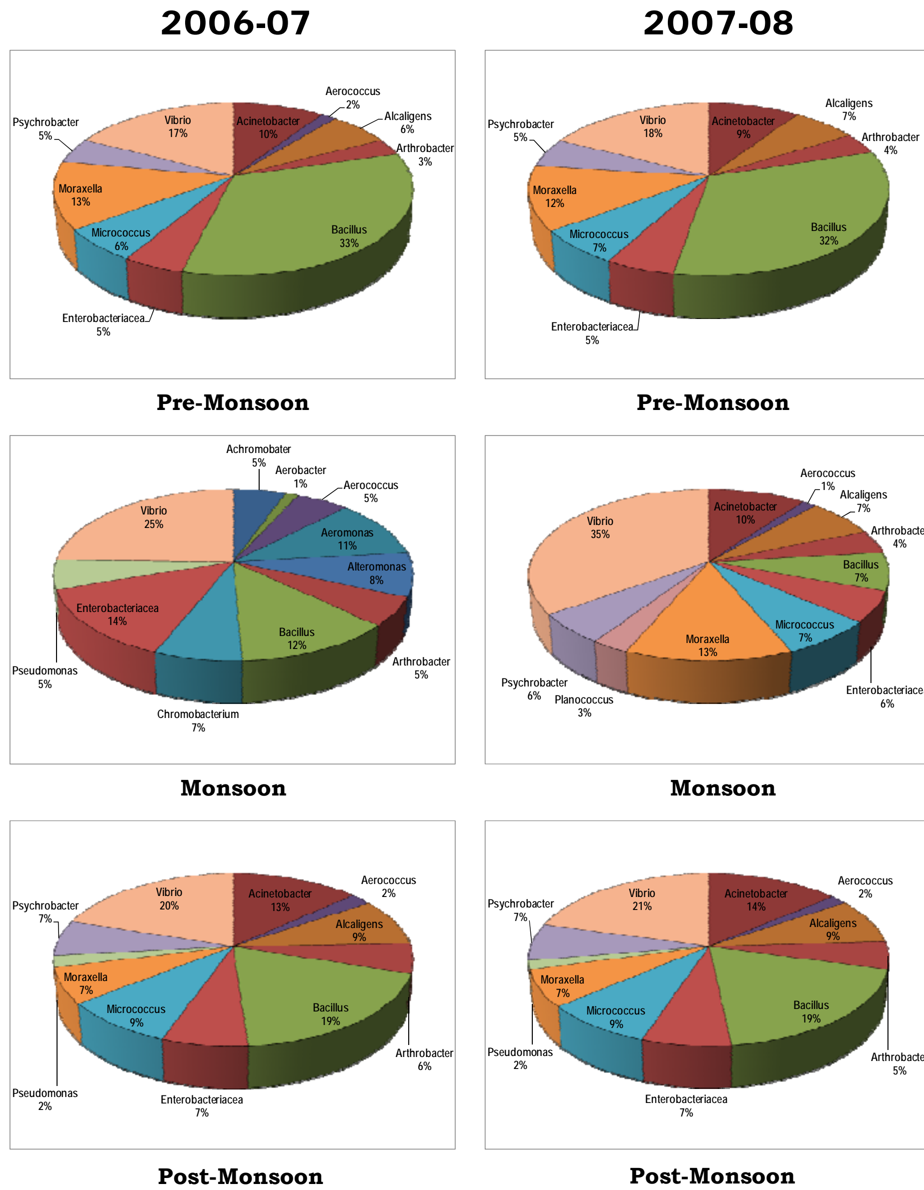


Fig.4.2f. Temporal variations in Heterotrophic bacterial composition at the estuarine station Azheekode during 2006-07 and 2007-08

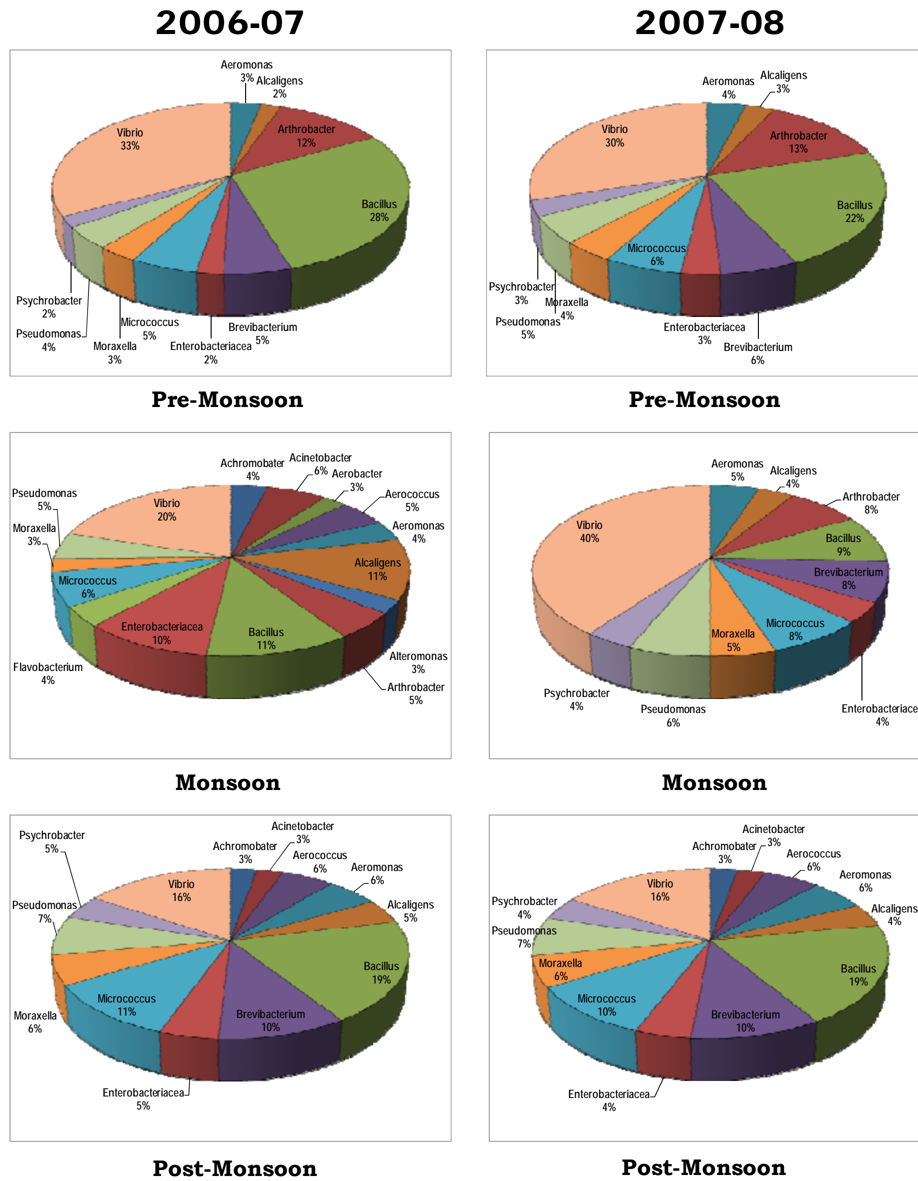


Fig.4.2g. Temporal variations in Heterotrophic bacterial composition at the estuarine station Fort Kochi during 2006-07 and 2007-08

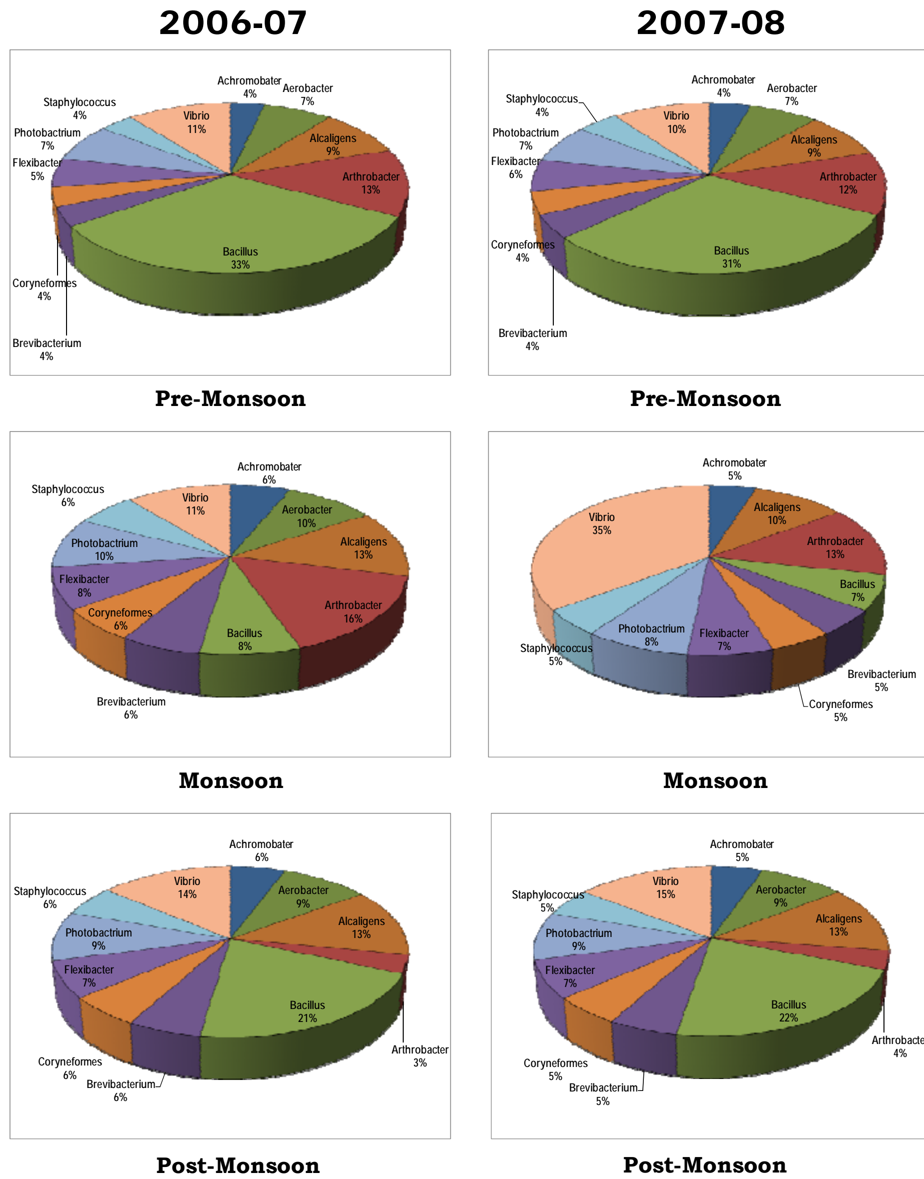


Fig.4.2h. Temporal variations in Heterotrophic bacterial composition at the estuarine station Kavanad during 2006-07 and 2007-08

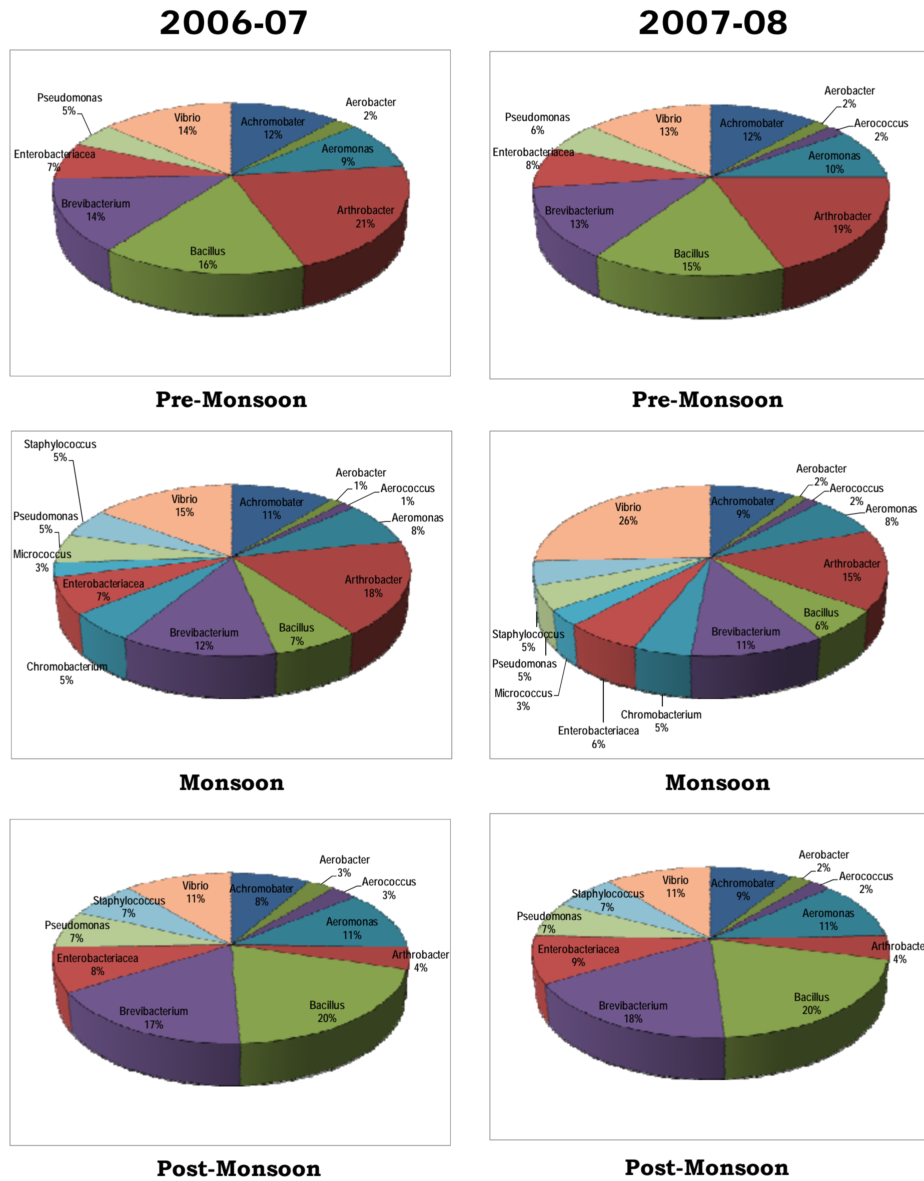


Fig.4.2i. Temporal variations in Heterotrophic bacterial composition at the mangrove station Dharmadom during 2006-07 and 2007-08

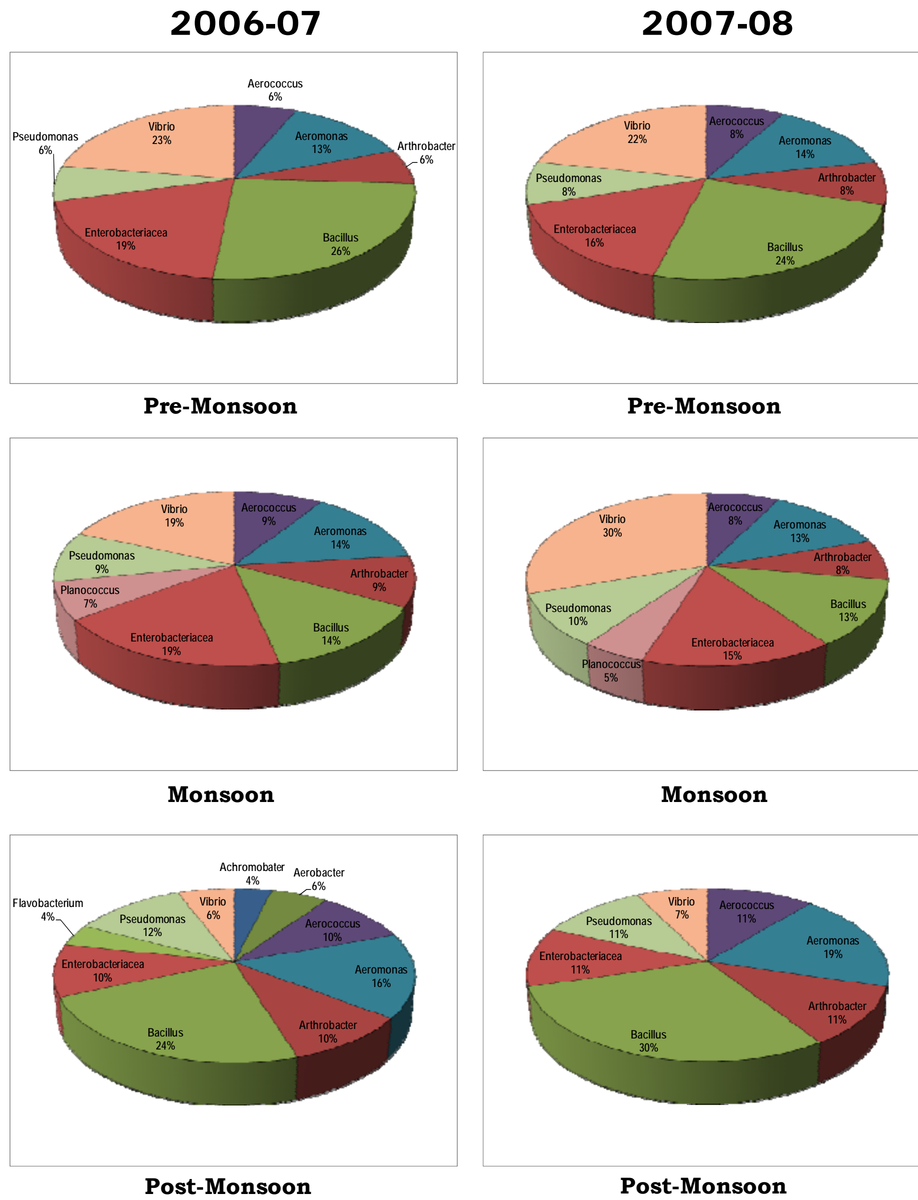


Fig.4.2j. Temporal variations in Heterotrophic bacterial composition at the mangrove station Kadalundi during 2006-07 and 2007-08

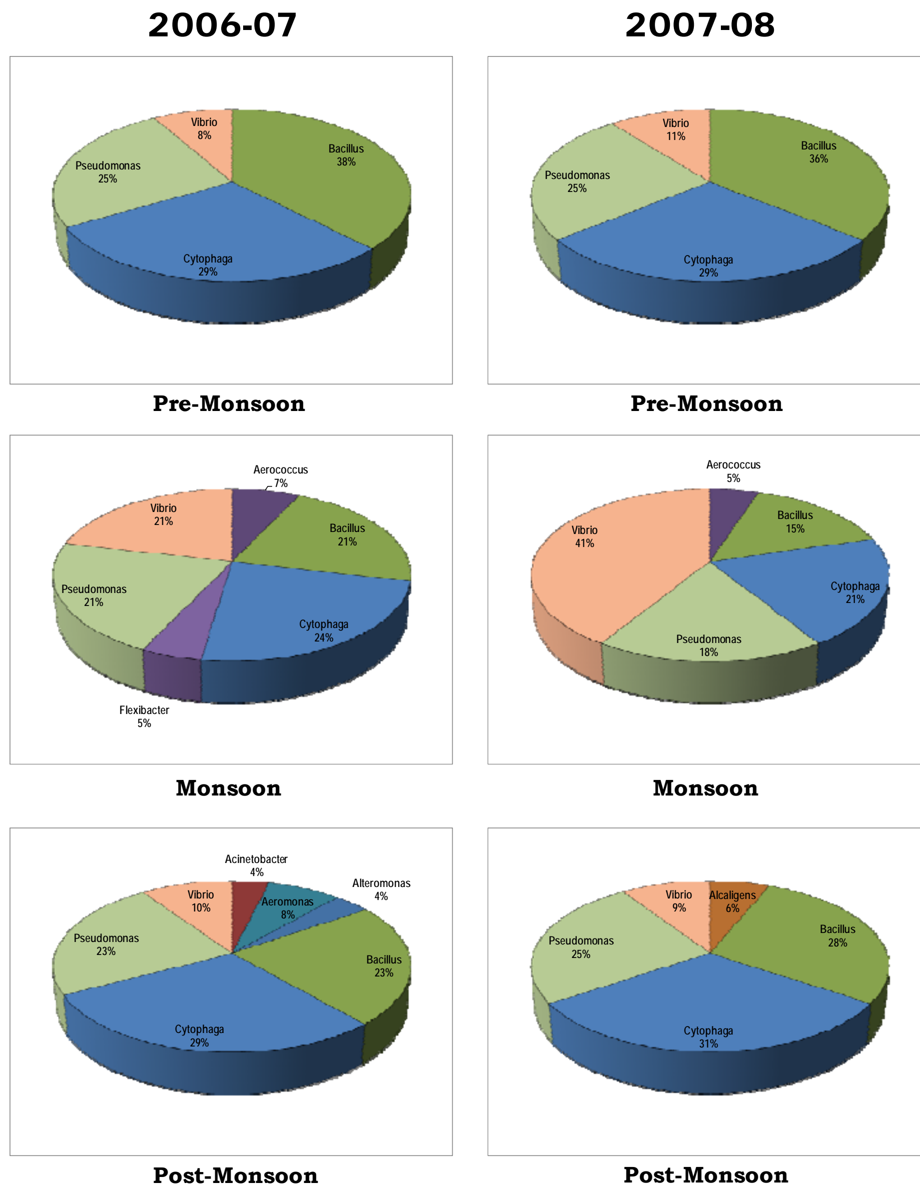


Fig.4.2k. Temporal variations in Heterotrophic bacterial composition at the mangrove station Puduvaipu during 2006-07 and 2007-08

4.3.1.2. Spatio-temporal variation of heterotrophic bacterial generic composition during 2007-08

In the overall percentage dominance of heterotrophic bacteria isolated during 2007-08, *Vibrio* (22.36%) was the most dominant genus, followed by *Bacillus* (18.67%), Enterobacteriaceae (6.91%), *Arthrobacter* (6.82%), *Pseudomonas* (6.77%), *Aeromonas* (5.42%), *Alcaligenes* (4.62%), *Achromobacter* (3.73%), *Micrococcus* (3.08%), *Brevibacterium* (2.80%), *Psychrobacter* (2.52%), *Aerococcus* (2.15%), *Acinetobacter* (2.01%), *Cytophaga* (1.73%), *Moraxella* (1.68%), *Aerobacter* and *Corynebacterium* (1.63% each), *Chromobacterium* (1.12%), *Flexibacter* and *Staphylococcus* (0.98%), *Alteromonas* (0.84%), *Photobacterium* (0.70%), *Flavobacterium* (0.56%) and *Planococcus* (0.28%, Fig. 4.3).

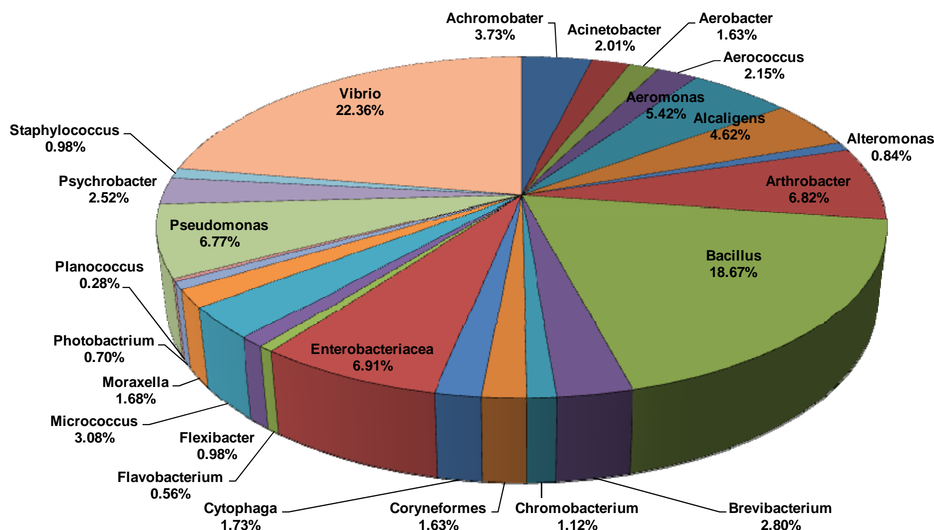


Fig. 4.3. The overall generic composition of culturable heterotrophic bacteria isolated during 2007-08

As observed in 2006-07, the coastal stations Kodikkal (Fig.4.2a), Punnapra (Fig.4.2b) and Vadi (Fig.4.2c) had a higher dominance of *Bacillus* during pre monsoon (Kodikkal – 25%; Punnapra – 25%; Vadi – 29%) and post monsoon (Kodikkal – 23%; Punnapra – 22%; Vadi – 25%), and *Vibrio* was found to be most dominant genus during monsoon (Kodikkal – 27%; Punnapra – 30%; Vadi – 34%). Except Fort Kochi (Fig. 4.2g), all the estuarine stations, namely, Mahe (Fig.4.2d),

Balathuruth (Fig.4.2e), Azheekode (Fig.4.2f) and Kavanad (Fig.4.2h), had a higher dominance of *Bacillus* during pre monsoon (Mahe – 33%; Balathuruth – 30%; Azheekode – 32%; Kavanad – 31%), while *Vibrio* was dominant in all the stations during monsoon (Mahe – 34%; Balathuruth – 34%; Azheekode – 35%; Fort Kochi – 40%; Kavanad – 35%). In the post monsoon season, *Bacillus* dominated Mahe (17%), Balathuruth (20%) and Kavanad (22%), while *Vibrio* was the dominant genus in Azheekode (21%) and Fort Kochi (16%). In the mangrove stations, *Bacillus* was the dominant strain in two stations each during pre monsoon (Kadalundi – 24%; Puduvaipu (36%) and post monsoon (Dharmadom – 20%; Kadalundi – 30%), while *Vibrio* dominated all the stations during monsoon (Dharmadom – 26%; Kadalundi – 30%; Puduvaipu – 41%). Varying from the common trend, Dharmadom (Fig. 4.2i) was dominated by *Arthrobacter* during pre monsoon (19%). As observed in 2006-07, the mangrove station Puduvaipu (Fig. 4.2k) had a relative abundance of *Cytophaga* during post monsoon (31%).

4.3.2. Variations in bacterial morphotypes of heterotrophic bacteria isolated during the study

4.3.2.1. Spatio-temporal variations of bacterial morphotypes during 2006-07

Among the heterotrophic bacteria isolated during 2006-07, Gram-negative forms (GN) were relatively abundant, being grouped under 17 genera, while Gram-positive forms (GP) contributed only 7 genera (Fig. 4.4). The percentage compositions of the Gram-positive and Gram-negative bacteria isolated from the various stations during the three different seasons are shown in Figs. 4.5. and 4.6, respectively.

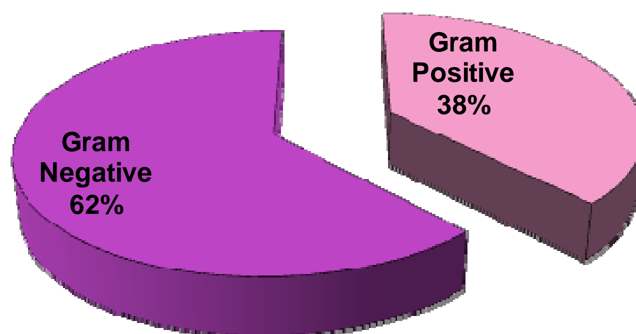


Fig. 4.4. Total percentage composition of gram Positive and Gram negative bacteria isolated during 2006-07

Among the gram positive bacteria, during pre monsoon, *Bacillus* was the dominant genera in all the stations, except Dharmadom (mangrove station). Dharmadom exhibited a higher representation of *Arthrobacter* (41%), closely followed by *Bacillus* (32%) and *Brevibacterium* (27%). Corynebacterium was mostly absent or scanty in most of the stations, with the highest occurrence being 25% at the coastal station, Vadi. *Micrococcus* was totally absent in the mangrove stations. *Staphylococcus* was present only in two stations, namely, the coastal station of Kodikkal (5% of total constitution) and the estuarine station of Kavanad (6% of total constitution).

During post monsoon also, a similar scenario was observed, with *Bacillus* being the dominant genera in all the stations. *Arthrobacter* was the second dominant genera in many of the stations, with the highest occurrence at the estuarine station of Mahe (36%). Corynebacterium was not present in any of the mangrove stations. It was found in one station each from the coastal and estuarine ecosystems, viz. coastal station Vadi (24%) and estuarine station Kavanad (14%). As in the case of pre monsoon, *Micrococcus* was totally absent in the mangrove stations. Staphylococcus formed 14% of the generic composition in the estuarine station Kavanad and the mangrove station Dharmadom, while it was not represented in any of the coastal stations.

The monsoon season exhibited a totally different scenario in all the three ecosystems. Among the 11 stations, five were dominated by *Arthrobacter* and four stations were dominated by *Bacillus*. There was equal dominance (25% each) of *Arthrobacter* and *Bacillus* at the coastal station Punnapra, while the other two coastal stations had a clear dominance of *Arthrobacter* over the other genera. The estuarine station Mahe experienced an exceptional dominance of *Corynebacterium* (62%). As for the other estuarine stations, *Arthrobacter* dominated Balathuruth (60%) and Kavanad (37%), while *Bacillus* dominated Azheekode (53%) and Fort Kochi (41%). Among the mangrove stations, Dharmadom was dominated by *Arthrobacter* (38%), while *Bacillus* dominated Kadalundi (43%) and Puduvaipu (75%).

Among the Gram-negative (GN) bacteria, during pre monsoon *Vibrio* was the dominant genera in all the stations, except the mangrove station Puduvaipu, which was dominated by *Cytophaga* (47%) and *Pseudomonas* (40%). Of the 17 GN genera isolated during this season, the coastal stations were represented by all the genera, except for *Moraxella* and *Photobactrium*, which were totally absent in these stations. The estuarine stations had representatives of 14 genera present in one or more of the stations, while *Cytophaga, Flavobacterium* and *Planococcus* were not present in any of the stations. The mangrove stations had the least representatives of the GN group, with only 11 genera being present in one or more of the stations. *Acinetobacter, Alcaligenes, Alteromonas, Chromobacterium, Flavobacterium* and *Planococcus* were totally absent from the mangrove stations.

During monsoon, *Vibrio* was the dominant genera in the coastal and estuarine stations, except for Kavanad, which has a relatively higher dominance of *Alcaligenes* (22%). Among the mangrove stations, Dharmadom was dominated by *Vibrio* (28%), and Kadalundi had an equal dominance of *Vibrio* and *Enterobacteriaceae* (28% each), while Puduvaipu was dominated by *Cytophaga* (33%) and *Pseudomonas* (30%), as observed in the pre monsoon season. However, *Vibrio* was also on par with *Pseudomonas*, representing 30% of the total genera at this station. Of the 17 genera isolated during the monsoon season, all the genra, except *Planococcus* were recorded in the estuarine stations and 13 genera were present in the coastal stations, while

mangrove stations had the least representation, with only 10 genera being recorded from these stations.

In the post monsoon season, the coastal stations were dominated by *Vibrio*, eventhough Kodikal had an equal dominance of *Vibrio* and Enterobacteriaceae (19% each). All the estuarine stations, except Mahe, had a clear dominance of *Vibrio*. At Mahe, *Alcalige*nes was the dominant genus (25%). The three mangrove stations exhibited significant differences in % composition of GN bacteria. Kadalundi was dominated by *Aeromonas* (28%), while Dharmadom had an equal dominance of *Aeromonas* and *Vibrio* (23% each). Puduvaipu, as observed during the other two seasons, had a clear dominance of *Cytophaga* (38%) followed by *Pseudomonas* (30%). Only 16 genera were recorded during this season, with *Planococcus* being absent in all the coastal, estuarine and mangrove stations. Of the 16 genera, 14 and 13 each were present in the coastal and estuarine stations, respectively, while mangrove stations had the least GN group representatives (9 genera).

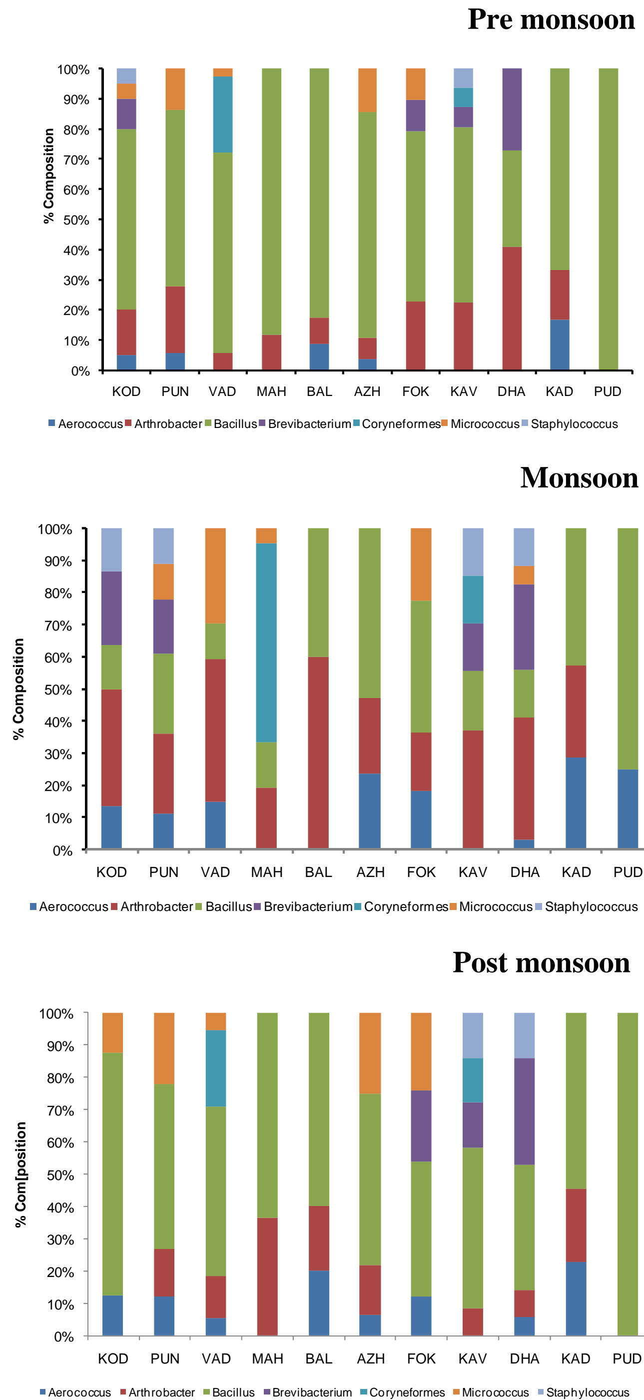
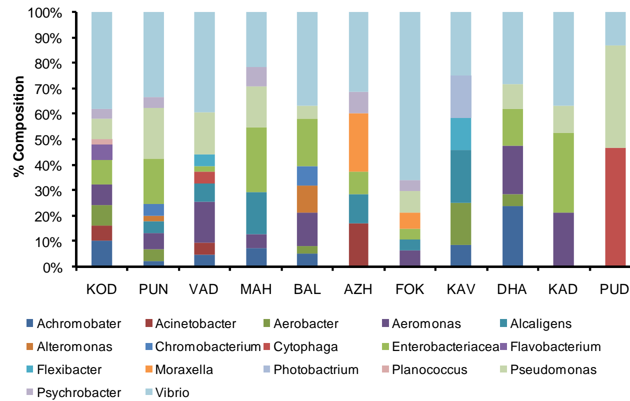
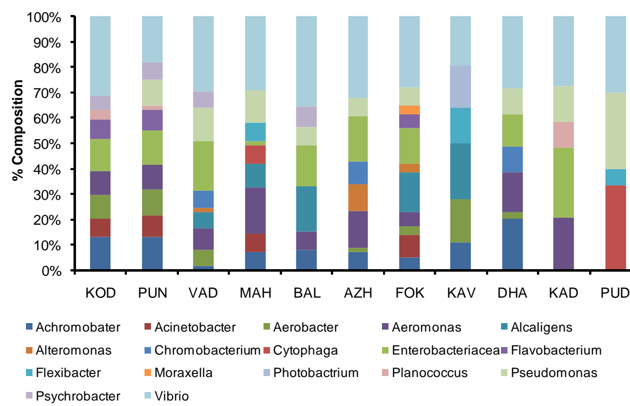


Fig. 4.5. Percentage composition of heterotrophic bacterial genera belonging to Gram-positive (GP) group isolated from the various stations during 2006-07

Pre monsoon



Monsoon



Post monsoon

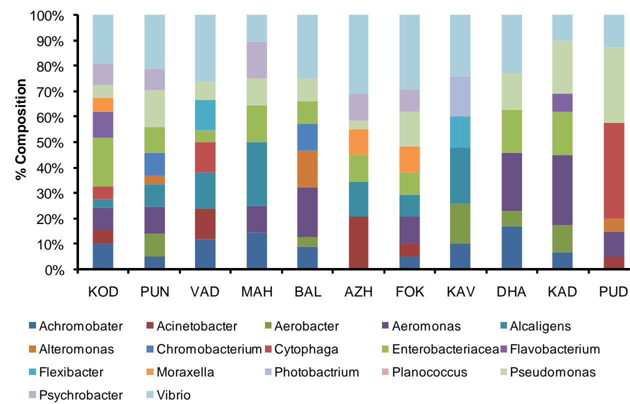


Fig. 4.6. Percentage composition of heterotrophic bacterial genera belonging to Gram-negative (GN) group isolated from the various stations during 2007-08

4.3.2.2. Spatio-temporal variations of bacterial morphotypes during 2007-08

During 2007-08 also, of the total heterotrophic bacteria isolated, Gram-negative forms (GN) were relatively abundant, being grouped under 17 genera, while Gram-positive forms (GP) contributed only 7 genera (Fig. 4.5). In the overall percentage dominance also, the GN *Vibrio* (22.36%) was the dominant genus compared to the GP *Bacillus* (18.67%, Fig. 4.7). The percentage compositions of the isolated Gram-positive and Gram-negative bacteria in the respective stations during the various seasons are shown in Figs. 4.8. and 4.9, respectively.

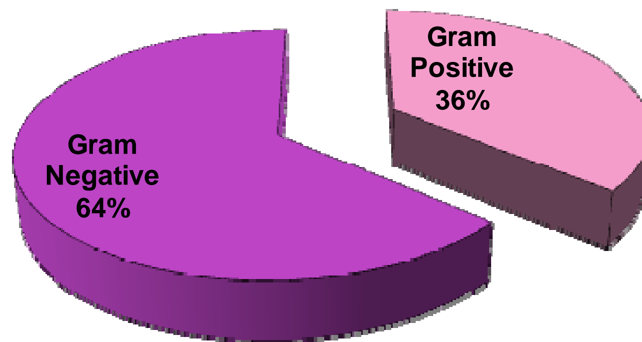


Fig. 4.7. Total percentage composition of gram Positive and Gram negative bacteria isolated during 2006-07

Among the GP bacteria, during pre monsoon, *Bacillus* was the dominant genera in all the stations, except Dharmadom (mangrove station). Dharmadom exhibited a dominance of *Arthrobacter* (41%), followed by *Bacillus* (31%) and *Brevibacterium* (27%). *Corynebacterium* was mostly absent or scanty in most of the stations, with the highest occurrence being 24% at the coastal station, Vadi. *Corynebacterium*, *Micrococcus* and *Staphylococcus* were totally absent in the mangrove stations.

During post monsoon, *Bacillus* was the dominant genera in all the stations. *Brevibacteriu*m and S*taphylococcus* were totally absent in the coastal stations and Corynebacterium and Micrococcus were not represented in the mangrove stations. There was a change in trend during the monsoon season with *Bacillus* being the dominant genera only in the estuarine stations of Balathuruth (54%) and Fort Kochi

(28%), the mangrove stations of Kadalundi (45%) and Puduvaipu (75%) and a co-dominant strain with Micrococcus in the estuarine station of Azheekode (36% each). Among coastal stations, *Arthrobacter* was the dominant genus (45%) at Punnapra and it was co-dominant with *Bacillus* (25% each) at Kodikal, while Corynebacterium was the dominant genus (63%) at Vadi. During this season, except for the absence of Corynebacterium in the mangrove stations, all the other genera were present in one or more stations of the three different ecosystems studied.

Vibrio was the dominant GN genus in most of the stations during 2007-08. During the pre monsoon season, *Vibrio* dominated all the three ecosystems, except for the estuarine station Mahe and the mangrove station Puduvaipu. At Mahe, Enterobacteriaceae (25%) had a higher % composition than *Vibrio* (22%). As observed in 2006-07, the mangrove station Puduvaipu exhibited the highest dominance of Cytophaga (44%) followed by *Pseudomonas* (39%). Of the 17 genera isolated, Moraxella and Photobacterium were totally absent in the coastal stations and Cytophaga, Flavobacterium and Planococcus were not present in any of the estuarine stations. The mangrove stations had the least diversity with only seven (Achromobacter, Aerobacter, *Aeromonas*, Cytophaga, Enterobacteriaceae, *Pseudomonas* and *Vibrio*) out of the 17 genera being present in these stations.

During monsoon, *Vibrio* was the dominant genera in the coastal, estuarine and mangrove stations. Though Puduvaipu was dominated by *Vibrio* unlike in 2006-07, it had Cytophaga (26%) as the next dominant genus, followed by *Pseudomonas* (23%). Of the 17 genera isolated during the monsoon season, all the genra, except Planococcus were recorded in the estuqarine stations and 13 genera were present in the coastal stations, while mangrove stations had the least representation, with only 10 genera being recorded from these stations.

In the post monsoon season, *Vibrio* was the dominant genus in the coastal stations, Punnapra (22%) and Vadi (25%) and it was co-dominant with Enterobacteriaceae (19% each) at Kodikal. As observed in 2006-07, all the estuarine stations, except Mahe, had a clear dominance of *Vibrio*. Mahe had the highest percentage abundance of Alcaligene genus (31%). Among the mangrove stations,

Dharmadom had a co-dominance of *Vibrio* and *Aeromonas* (23% each), while *Aeromonas* had clear dominance at Kadalundi (38%). Keeping in with the previous trend, Puduvaipu had a dominance of Cytophaga (43%) followed by *Pseudomonas* (35%). This time also, only 16 genera were recorded during this season, owing to the absence of Planococcus in all the coastal, estuarine and mangrove stations.

Only 16 genera were recorded during this season, with Planococcus being absent in all the coastal, estuarine and mangrove stations. Of the 16 genera, 14 and 13 each were present in the coastal and estuarine stations, respectively. Photobacterium and Planococcus were not present in any of the coastal stations. Likewise, cytophaga, Flavobacterium and Planococcus were absent in the estuarine stations. The mangrove stations had the least GN group representatives (9 genera) during this season. Acinetobacter, Alteromonas, Chromobacterium, Flavobacterium, Flexibacter, Moraxella, Photobacterium, Planococcus and Psychrobacter were not represented in any of the mangrove stations.

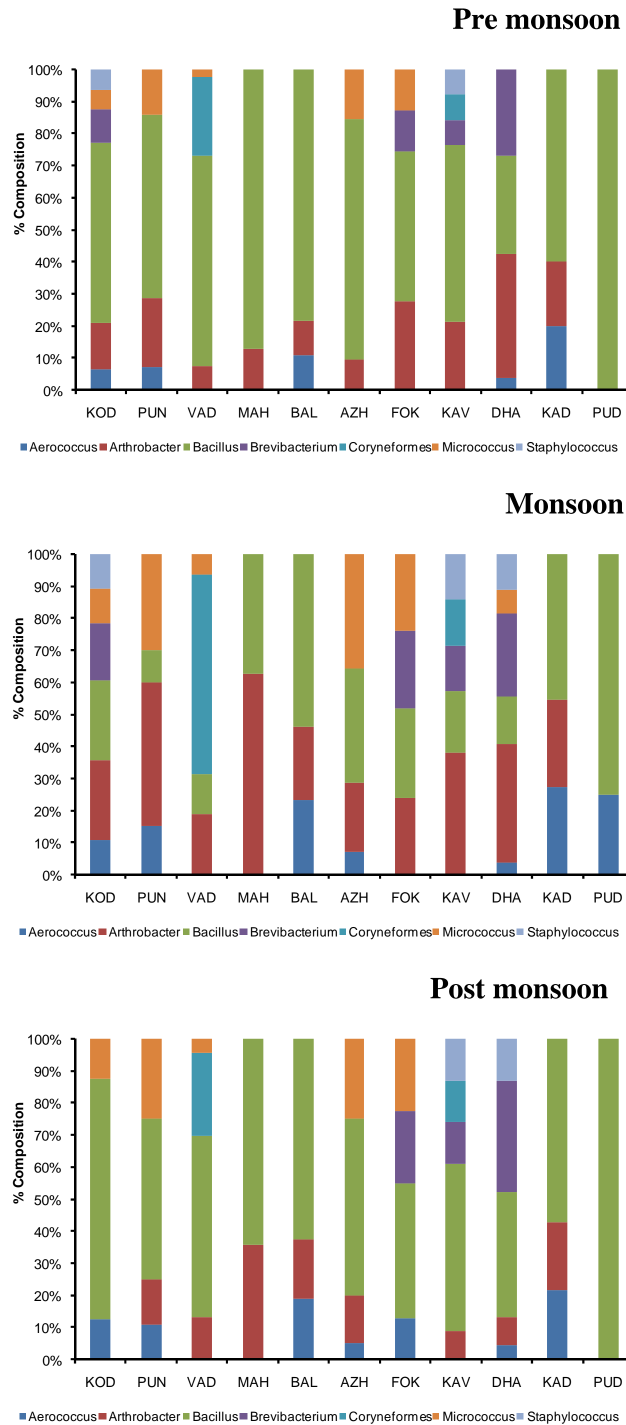


Fig. 4.8. Percentage composition of heterotrophic bacterial genera belonging to Gram-positive (GP) group isolated from the various stations during 2007-08

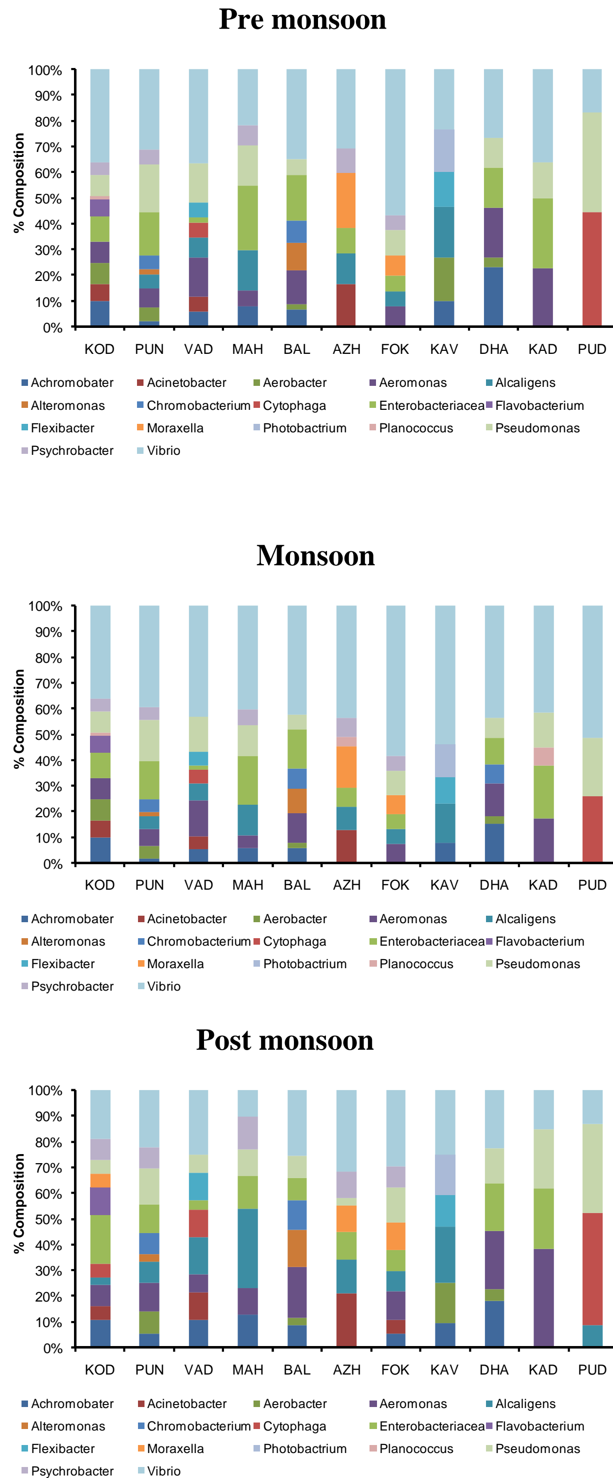


Fig. 4.9. Percentage composition of heterotrophic bacterial genera belonging to Gram-negative (GN) group isolated from the various stations during 2007-08

4.3.3. Statistical analysis

4.3.3.1. Cluster analysis and Non-parametric MDS ordination

The dendrogram plotted using the Bray Curtis similarity of measure and group average sorting of the bacterial generic diversity of the stations during pre monsoon, monsoon (MON) and post monsoon (POM) seasons of 2006-07 (Fig.4.10) indicated two major groups at 80% similarity level in PRM, 85% similarity level in MON and 90% similarity level in POM season. Group 1 consisted of the mangrove stations Kadalundi and Puduvaipu, while Dharmadom and all the coastal and estuarine stations formed the second group. Group 2 in PRM formed two sub-groups at 90% similarity level, while group 2 of MON and OM formed further sub-groups at 96% similarity level (Fig.4.10). The Multidimensional Scaling (MDS) plot of the three seasons during 2006-07 also showed similar grouping among the stations based on bacterial generic diversity (Fig.4.11). During 2007-08 also, the hierarchial cluster dendrogram indicated delineation of two groups at 80% similarity level during PRM and 85% similarity level during MON and POM seasons (Fig.4.12). The MDS plot of the three seasons during 2007-08 also showed similar grouping among the stations based on bacterial generic diversity (Fig.4.13).

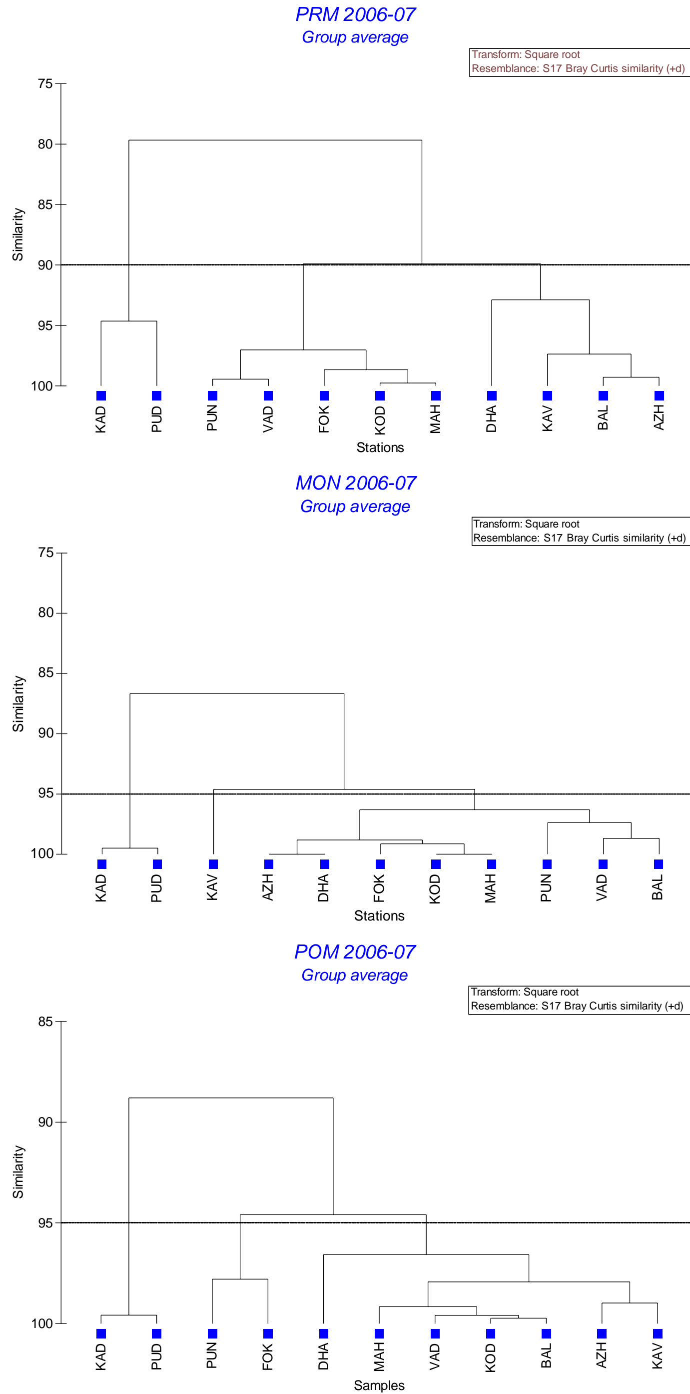


Fig.4.10. Hierarchial Cluster dendrogram plotted based on similarity and dissimilarity of the sampling stations in relation to the bacterial diversity during 2006-07

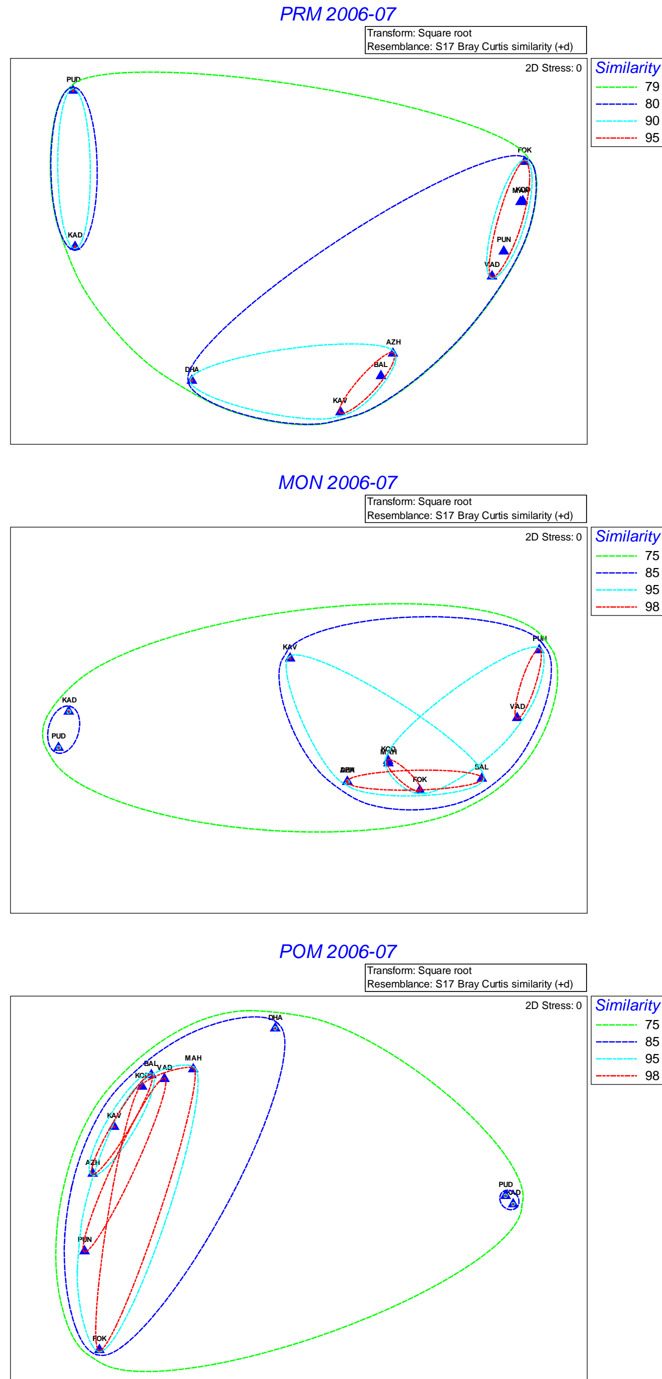


Fig.4.11. Multidimensional Scaling (MDS) plotted based on similarity and dissimilarity of the sampling stations in relation to the bacterial diversity during 2006-07

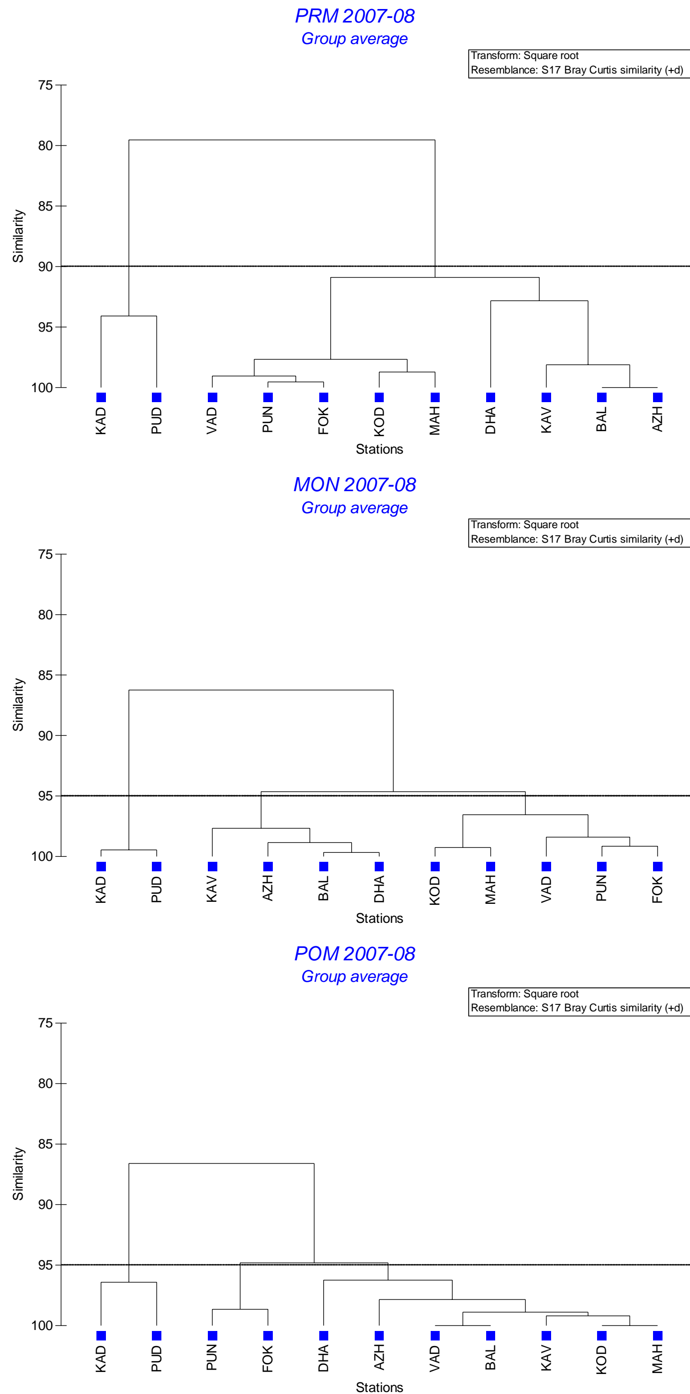


Fig.4.12. Hierarchial Cluster dendrogram plotted based on similarity and dissimilarity of the sampling stations in relation to the bacterial diversity during 2007-08

PRM 2007-08

MON 2007-08

POM 2007-08

Fig.4.13. Multidimensional Scaling (MDS) plotted based on similarity and dissimilarity of the sampling stations in relation to the bacterial diversity during 2007-08

4.3.3.2. K-Dominance curve

The K-dominance curve plotted for the three seasons during 2006-07 indicated that pre monsoon had higher dominance compared to monsoon and post monsoon. It was observed that a single genus in pre monsoon contributes to nearly 28% of the cumulative abundance, while a single genus from monsoon and post monsoon contributed to only 20% of the cumulative abundance. However, during 2007-08, monsoon had the highest dominance, with a single species contributing to approximately 33% of the cumulative dominance, followed by pre monsoon and post monsoon with approximately 26% and 20% contributions, respectively.

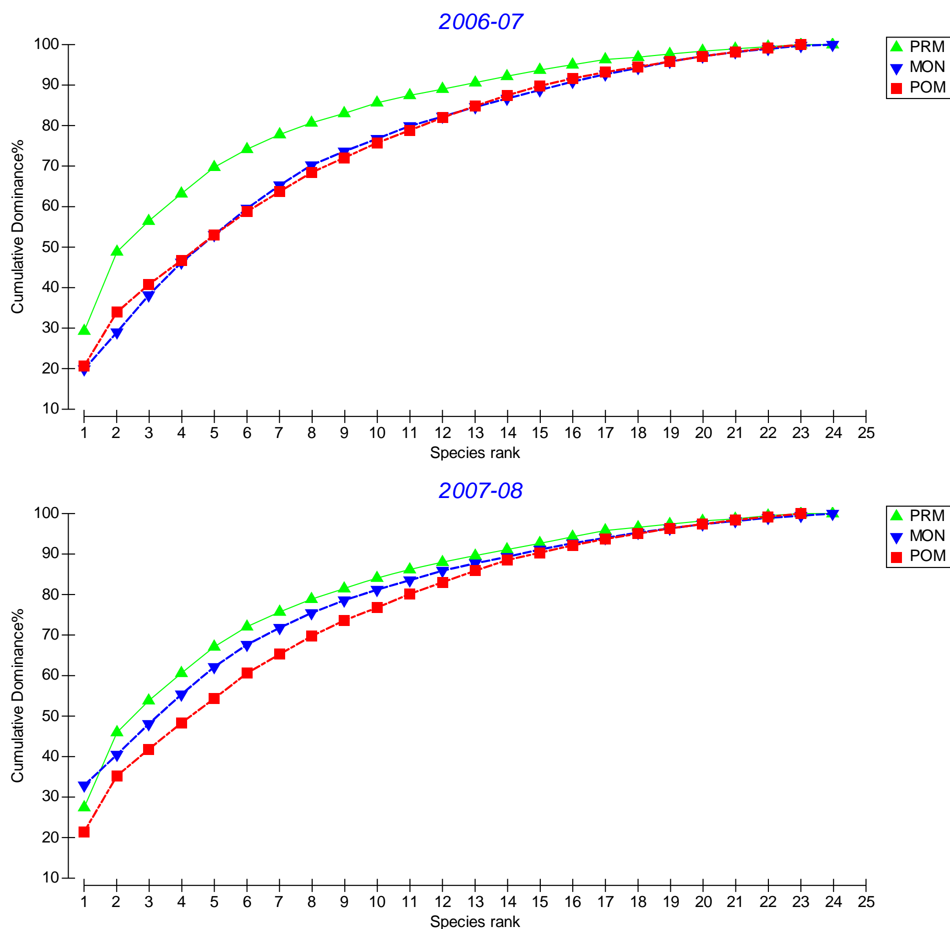


Fig.4.14. K-dominance curve plotted for the different seasons based on the bacterial generic diversity during 2006-07 and 2007-08

4.3.3.3. Diversity Index Measures

The diversity indices of different seasons during 2006-07 and 2007-08 are presented in Table 4.1. During 2006-07, Shannon-Wiener diversity (H'; loge) was higher in monsoon (2.79), followed by post monsoon (2.76) and pre monsoon (2.42). Similarly, dominance (1-Lambda') was also higher in monsoon (0.92), followed by post monsoon (0.91) and pre monsoon (0.86). Pielou's evenness (J') was equal for monsoon and post monsoon (0.88 each), while pre monsoon had a J' value of 0.76. The Species richness (d) was found to be highest in pre monsoon (3.50), followed by monsoon (3.45) and post monsoon (3.25). During 2007-08, Shannon-Wiener diversity (H'; loge), Species richness (d) and Pielou's evenness (J') was highest in post monsoon, followed by monsoon and pre monsoon. Dominance (1-Lambda') was also highest in post monsoon (0.91), but was followed by pre monsoon (0.87) and monsoon had the lowest value (0.86).

Table 4.1. Diversity indices of different seasons during 2006-07 and 2007-08

	2006-07			2007-08		
S	24.00	24.00	23.00	24.00	24.00	23.00
N	711.00	792.00	877.00	832.00	753.00	557.00
Species richness d	3.50	3.45	3.25	3.42	3.47	3.48
Pielou's evenness J'	0.76	0.88	0.88	0.78	0.80	0.87
Species diversity H'(loge)	2.42	2.79	2.76	2.49	2.54	2.73
Species dominance 1-Lambda'	0.86	0.92	0.91	0.87	0.86	0.91

4.4. Discussion

The sediment microbiota is an inevitable component of the marine ecosystem. The understanding of their critical role in basic ecosystem processes such as primary production and remineralization of organic material has led to the prioritization of benthic microbial research (Decho, 2000; Hewson *et al.,* 2007; Raulf *et al.,* 2015).

Eventhough lot of research has been carried out on the characterisation of bacterial community structures, a large segment of the marine sediment bacteria are yet to be defined. However, of late, there has been a growing understanding of the structure of shallow sediment microbiota and of their role in the dynamics of coastal sediment ecosystems (Böer *et al.,* 2009). Moreover, the knowledge of the bacterial community of a given environment provides an insight into the habitat characteristics, since bacteria respond quickly to changes in the abiotic parameters (Stabili and Cavallo, 2004). The present study highlights the distribution of culturable heterotrophic bacteria in the various coastal ecosystems along the south-west coast of Arabian Sea by qualitative and quantitative description of their generic diversity and community variations occurring in a spatial and temporal scale.

Since the first recognition of microorganisms, scientists have devised classification schemes with the goal of systematically identifying species in an evolutionary or phylogenetic context (Clarke, 1985). But, it is a well-known fact that even today only a small fraction of the natural microbiota can be cultured using laboratory media. According to reports, only 0.1-1 % of marine bacteria are culturable using classic cultivation methods, while culture-independent techniques like microautoradiography, direct viable count technique and flow cytometry have revealed that upto 50% (upto 90% in some cases) of the bacterial cells are metabolically active (Amann *et al.*, 1995; Pace, 1997; Gerdts and Luedke, 2006). Due to their small size, bacteria have a limited range of morphological characteristics. However, they exhibit a wide range of biochemical diversity in their metabolism and cell structure which has proved to be a useful cue for the taxonomic identification of some groups. Since a proper scheme of analysis covering the total microbial diversity is still not in place, the classic techniques involving isolation and cultivation of culturable heterotrophic bacteria is still followed in microbial ecology studies. Molecular techniques like ribosomal RNA gene sequences, low molecular weight RNA profiles and DNA hybridizations are used for in-depth studies of selected bacterial strains (Pace *et al.,* 1986; Emerson *et al.,* 2008). Therefore, in the present study, a culture-based approach was adapted to determine the bacterial generic diversity in the benthic realm of selected coastal, estuarine and mangrove ecosystems along the Kerala coast.

It has been reported that about 90% of marine heterotrophic bacteria are gram-negative forms since the cell wall in these organisms is better adapted for survival in the marine environment (Das et al., 2006). In the current study also, gram negative forms were more dominant compared to the gram positive forms during 2006-07 (GN – 62%; GP – 38%) and 2007-08 (GN – 64%; GP – 36%). This is in contrast to earlier reports where high concentration of GN was recorded from the marine water column, but GP was found to dominate the sediment (Chandramohan *et al.,* 1987; Cavallo *et al.,* 1999; Loka Bharathi and Nair, 2005). The observed dominance of GN forms during the present study can be attributed to the samples being collected from the surface layer of near-shore ecosystems. In marine systems, GN bacteria being the initial colonizers successfully thrive in dynamic ecosystems with instabilities in physico-chemical properties and the GP forms are found to colonize the relatively undisturbed deeper sediment layers (Genron *et al.,* 1984). Ramya *et al.* (2013) also observed an abundance of GN bacteria over GP forms from the shelf sediments of south eastern Arabian Sea.

Although GN forms dominated the samples, in the overall percentage dominance of various genera, the GP *Bacillus* (19.12%) was the most dominant genus, followed by the GN *Vibrio* (17.39%) in 2006-07, while there was a reverse trend in 2007-08, wherein, the GN *Vibrio* (22.36%) was the most dominant genus, followed by the GP *Bacillus* (18.67%). Also, 17 genera belonging to GN group were isolated during this study, while GP group consisted of only 7 genera. The occurrence of *Bacillus* spp. in the marine environment has been well documented (Ivanova *et al.,* 1999). This genus constitutes a diverse group of rod-shaped, Gram-positive bacteria, belonging to the phylum Firmicutes. They are capable of aerobically forming resistant endospores, making them ubiquitous in the environment. Divya *et al.* (2010) has reported a high proportion of Firmicutes in the sediment in the Arabian Sea. The genus *Bacillus* has already been reported to be dominant in the sediment samples of Bay of Bengal and Arabian sea (Palaniappan and Krishnamurthy, 1985; Ramya *et al*., 2013; Jacob *et al*., 2013). Stabili and Cavallo (2011) also obtained similar results during their studies on the southern Adriatic Sea Italian coasts. The Bacillales are known to survive under diverse

conditions like seawater and sediment, which further supports their ubiquitous nature. It is presumed that the majority of the *Bacillus* spp. reported are terrestrial bacteria blown or deposited as spores into marine sediments (Ivanova et al., 1999). But the fact that several species of *Bacillus* require seawater for growth indicates their obligate marine nature (Imada *et al.*, 1998; Gugliandolo *et al.,* 2003). It could also be possible that the terrestrial *Bacillus* species got transported into the marine environment by land-runoff and through evolutionary adaptation developed as obligate marine forms (Liu *et al.,* 2013). This could also be the possible reason behind the relative dominance of *Bacillus* in the current study, since the samples were collected from near-shore environments. The dominance of *Vibrio* over *Bacillus* in 2007-08 can be attributed to the relatively high abundance of *Vibrio* in all the stations during monsoon season of 2007-08, possibly due to increased land runoff and/or sewage discharge. Further, bacterial metabolism is such that, if a particular group is the dominant bacterium in the sewage discharges, it can compete and rapidly outgrow the native microflora leading to increased levels of indicator bacteria in natural water bodies (Nagvenkar and Ramaiah, 2009). The same could be applied to marine sediments as well. *Vibrio*s, as such, have been reported to be highly abundant in estuaries, marine coastal waters and sediments (Ortigosa *et al.,* 1994; Barbieri *et al.,* 1999; Urakawa *et al.,* 2000). The presence of Enterobacteriaceae in considerable percentage during 2006-07 (7.31%) and 2007-08 (6.91%) further indicates the possibility of sewage contamination in the near-shore waters (Munn, 2011).

Other heterotrophic bacteria isolated during this study period included *Arthrobacter*, *Pseudomonas*, *Aeromonas, Alcaligenes, Achromobacter, Micrococcus, Brevibacterium, Aerococcus, Psychrobacter, Aerobacter, Acinetobacter, Cytophaga, Corynebacterium, Staphylococcus, Chromobacterium, Moraxella, Flexibacter, Alteromonas, Flavobacterium, Photobacterium and Planococcus*. The GN aeromonads are considered to be one of the most abundant bacteria found in the aquatic environments (Cavallo *et al.,* 1999). During the current study also, *Aeromonas* was considerably abundant among the Gram-negative bacteria (5.92% and 5.42% during 2006-07 and 2007-08, respectively. This genus has been reported to be an autochthonous organism occurring in uncontaminated as well as sewage-

contaminated waters (Burke *et al.,* 1984). Also, *Aeromonas* is reported to be predominant in waters with high levels of faecal pollution, and it has therefore been claimed that the presence of aeromonads can assist in assessments and predictions of aquatic system deterioration or recovery (Venkateswaran and Natarajan, 1987; Araujo *et al.,* 1991). Considering the relatively high incidence of *Aeromonas* during the current study, anthropogenic influence, by way of sewage pollution in the near-shore coastal environments, cannot be ruled out.

Apart from *Bacillus*, another important group of Gram-positive forms found in the sediment bacterial community of the study area is the genus *Arthrobacter*. It has been reported to be one of the most important hydrocarbon-degrading bacterial groups, well-known for their capacity for degrading natural aliphatic and aromatic hydrocarbons and xenobiotics polluting the environment as a result of human activities (Eaton, 2001; Khasaeva *et al.,* 2007; Plotnikova *et al.,* 2011). The presence of *Arthrobacter* in significant amount during the current study (>6% of total bacterial abundance) indicates possible hydrocarbon pollution (Saadoun et al., 2008). *Corynebacterium* represented more than 1% of the microbial community during the present study. Maruyama *et al.* (1997) have reported the occurrence of non-spore forming Gram-positive rod shaped forms belonging to group *coryneforms* in the deep-sea sediments of the Pacific Ocean. Loka Bharathi and Nair (2005) observed that disturbance or bioturbation in the sediment substratum evoked an increase in the abundance of G-positive coryneforms among the deep-sea bacteria of Central Indian Ocean Basin. Another important group isolated during the study was *Micrococcus*. The occurrence of this genus in the coastal waters has been earlier reported by several researchers (Dhevendaran *et al.,* 1987; Chandrika and Nair, 1994). Among the GN forms, *Pseudomonas* represents a fraction of the total microbial flora characterized by high metabolic versatility, and it is known for its capacity to degrade a considerable quantity of synthetic organic compounds. This genus is often encountered in sea water, sediments, phytoplankton and zooplankton (Austin *et al.,* 1979; Nair and Simidu, 1987). Under certain conditions, such as organic matter in solution and temperature in the mesophilic range, the capacity of *Pseudomonas* for rapid growth in the absence of complex growth factors is responsible for its predominance (Palleroni, 1984). Members of the genus *Acinetobacter* are usually

isolated from water samples. They are capable of degrading aromatic compounds and their metabolic versatility is similar to that of the pseudomonads (Abd-el- haleen *et al.,* 2002). Organisms of the genus *Moraxella* are commonly isolated from animals. Maruyama *et al.* (1997) showed that among the psychrotrophs from deep-sea water there was abundance of *Moraxella* and deep-sea *Moraxella* were different from surface ones. *Moraxella* plays an important role in nitrogen cycling (Stolp, 1988). The genus *Chromobacterium* is ubiquitous in nature and is found in waters from polar regions to the tropics. *Flavobacterium* has been isolated from marine water, ocean sediments and foods; it tolerates low, but prefers higher temperatures and can grow in alkaline environments (Stolp, 1988). This genus has been reported to be involved in the degradation of pesticides and chitin. In marine environments, *Flexibacter* is abundant near to the shore, on seaweeds and on decaying sea animals. *Cytophaga* group occurrence indicates the supply of fresh macro aggregates (Riemann *et al.,* 2000). As naturally occurring bacteria *Cytophaga* spp. are capable of biodegrdation of natural polymers such as cellulose, pectin, keratin, agar and chitin. *Photobacterium* are usually found in marine environments. They may be free living in the water, or found associated with intestinal contents of marine animals and with the specialized luminous organs of fish (Baumann and Baumann, 1984). The genus *Flavobacterium* is one of the more commonly represented genera in the marine environment. This genus has been isolated from seawater and marine sediments by ZoBell (1946). *Flavobacterium* was also isolated by Ohwada (1980) from Atlantic Ocean. Nair *et al.* (1994) found that *Flavobacterium* was confined to the offshore stations in Indian Ocean. Further, it has also been suggested that *Flavobacterium* and *Alcaligenes* genera are common in the marine environment and undergo seasonal fluctuation (Seshadri *et al.,* 2002).

Determination of bacteriological indices enables assessment of the trophic status of the sediment biome and help in assessment of the extent of disturbance taking place in a given region. In the present study, the Shannon indices ranged from 2.42 to 2.79, which indicate rich species diversity. This is higher than the reported Shannon indices of 1.8 to 2.4 from Norwegian coastal waters (Larsen *et al.,* 2004) and higher than values reported from the Catalan coast (2.5–3.1; Schauer et al. 2000). Dave and Desai (2006) studied the microbial diversity from the west coast of India.

They found Shannon-Wiener index in the range of 0.048 - 1.334, evenness in the range of 0.070 - 0.829 and species richness in the range of 0.621 - 2.485. In the current study, Species richness ranged from 3.25 to 3.50, while Pielou's evenness ranged from 0.76 to 0.88. The diversity indices indicate only small variations in community structure between the stations and between the seasons. The *K*-dominance curve is a powerful tool for measuring abundance trends in communities over time. The steepest and most elevated curve shows the lowest diversity and the most perturbed system state (Rice 2000). In the current study, however, there was only slight variation between the K-dominance curves, indicating a relatively stable ecosystem.

Eventhough the temporal variations in bacterial generic diversity was negligible, cluster analysis and MDS plots on a spatial scale indicated the formation of two distinct groups, group one consisting of the mangrove stations, Kadalundi and Puduvaipu, and group two including Dharmadom and all the coastal and estuarine stations. However, the similarity level for delineation of the two groups was always greater than or equal to 80% during the three different seasons. This implies that even the stations grouped under separate clusters had a minimum of 80% similarity in the bacterial diversity. The distinct cluster formation by the two mangrove stations can be attributed to the least generic diversity in these stations. Among the mangrove stations, Dharmadom had relatively higher generic diversity and hence was found to group along with the coastal and estuarine stations. The increase in bacterial diversity at Dharmadom compared to Kadalundi and Puduvaipu can be attributed to the strategic location of Dharmadom in close proximity to estuarine and coastal sediments. The current observation of low bacterial diversity in mangrove ecosystems is in contrast to earlier reports of mangroves being hotspots for microbial diversity (Alongi, 1988). Microbes engage in biogeochemical cycles and supply plants and animals with primary nutritional sources, thereby perpetuating microbial diversity and activity as the fundamental aspects for the productivity, conservation, and recovery of mangroves (Kathiresan and Bingham, 2001; dos Santos et al., 2011). However, a recent study indicated a higher functional redundancy in the oxidized top layer of marine sediments, where higher disturbance rates and higher availability of substrates may lead to the formation of a community of fast-growing generalists

(Boer et al., 2009). Functional redundancy is based on the observation that some species perform similar roles in communities and ecosystems, and may therefore be substitutable with little impact on ecosystem processes (Lawton and Brown 1993). In the present study, owing to the constant shifts experienced by the mangrove ecosystems (especially Kadalundi and Puduvaipu) as a result of anthropogenic influence, *Bacillus*, *Aerococcus, Enterobacteriaceae, Cytophaga* and *Pseudomonas* could have probably developed as generalists, causing functional redundancy, resulting in the overall reduction of bacterial genera contributing to community formation. On the whole, the current study contributes to the community structure assessment of the sediment bacteria of coastal, estuarine and mangrove ecosystems of the Kerala coast.

CHAPTER 5

HYDROLYTIC EXTRACELLULAR ENZYME PRODUCTION POTENTIAL OF ISOLATED BACTERIAL STRAINS

5.1. Introduction

Microorganisms are capable of exploiting a vast range of energy sources which subsequently equip them to adapt in varied habitats. They are the key regulators of basic ecosystem processes by contributing towards global elemental cycling. The oceanic sedimentary mineralization processes are believed to be occurring over the continental shelf where most of the organic matter breakdown is being undertaken by various forms of microorganisms and in particular by bacteria (Walsh, 1991; Jedrezejczak, 1999). The production and secretion of extracellular hydrolytic enzymes in sedimentary environment may have important biogeochemical implications, especially in organic biopolymer compound degradation, nutrient recycling and mobilization of the essential biogeochemical elements. In these nearshore continental shelf sediments various bacteria showing the capacity to decompose lipids, proteins and different substrates are abundant. The production of bacterial hydrolytic enzymes and their activity depends on the availability, distribution and concentration of organic substrates (Boetius, 1995).

Heterotrophic bacteria are considered as the key players in the processes of organic matter recycling, decomposition and mineralization in aquatic environments. The small and chemically simple compounds can be passively transported through bacterial membranes. In aquatic environments the substrates for bacterial utilization are dominated by high molecular weight particulate or dissolved organic matter. These complex substrates are hydrolyzed in the exterior environment of the bacterial cell into smaller sized molecules using the array of specific extracellular enzymes. This is often considered as a limiting step in nutrient cycling. The main sources of organic matter to the microbial loop are phytoplankton exudation and leakage of algal or bacterial cell material during grazing (Azam *et al.,* 1983). Benthic re-

suspension, plant and algal exudation and terrestrial or riverine inputs represent significant additional sources of organic matter, which can be used as carbon and energy sources (Williams and Jochem, 2006). The passive transport of chemically small and simple compounds through the bacterial cell wall and the cell membrane is selectively permeable to a size range (Amon and Benner, 1996). This assortment is made feasible due to the chemical composition and architecture of the bacterial cell wall. The wall composition of gram positive and gram negative bacteria only allows the transport of small molecules which can be used for rapid energy synthesis (Decad and Nicaido, 1976). It is now understood that the gram positive cell wall is not as restrictive, in terms of permeability in comparison with the gram negative counterparts. This is because the outer membrane of gram negative bacteria form channels between the outer membrane and the periplasmic space is made of porins whose geometry restricts the uptake limit to ~600 Da (Ghuysen and Hackenbeck, 1994). Marine sinking particles provide potential "hot spots" for microbial decomposition of organic matter (Azam, 1998; Azam and Long, 2001). Because the bacteria can only incorporate small molecules (<600 Da) via their cell-membrane; permeases, macromolecules and particles must be broken down to monomers prior to their incorporation (Weiss *et al.*, 1991). In marine environments the particulate organic matter (POM) and dissolved organic matter (DOM) are generally dominated by high-molecular-weight compounds (Salton, 1994; Labischinski and Maidhof, 1994).

It can be inferred that the bacterial extracellular enzymatic activity is regulated at the ecosystem level by various environmental factors that govern the integrity and balance of the particular environment. Whereas, at the micro-environment level it is the enzyme-substrate interactions that govern the flow of energy which in turn is dependent on the environmental factors. Owing to the global climate changes, the increase in the atmospheric concentration of CO_2 and other greenhouse gases has caused changes that have serious repercussions in ecosystem function and biodiversity. Since the microorganisms are generally able to respond very quickly to environmental changes because of their close contact with the surrounding environment and rapid growth, they are considered as intermediaries in important biogeochemical processes, namely decomposition and transformation of

organic matter, release of inorganic nutrients for higher trophic levels and detoxification of xenobiotics (Amon and Benner, 1994). Thus the bacterial enzyme activities have the potential to be used as characteristic features for understanding the biological responses to changing environmental conditions. For the transport across the outer membrane, the complex substrates must first be hydrolyzed outside the cell into smaller sized molecules so as to enable its passive entry into the cell (Chróst, 1991; Weiss *et al.,* 1991). This process is accomplished by extracellular enzymes which enable heterotrophic bacteria to obtain substrates suitable for incorporation from a diverse array of complex compounds (Arnosti, 2003). The extracellular degradation of complex molecules into easily assimilable units thus forms the rate limiting step in the global nutrient recycling and remineralization. So any factors disrupting the production or availability of extracellular enzymes will impact the entire remineralisation pathway (Arnosti, 2003). Thus the changes in the patterns of organic matter utilization by bacteria may also impose carbon cycle-mediated feedbacks on global climate (Bardgett *et al.,* 2008). The environmental regulation model of extracellular enzyme activity proposes that at the ecosystem level, enzyme production is mainly regulated by environmental factors such as temperature and that at the micro-environmental level the enzyme activity is mostly controlled by enzyme-substrate interactions such as inhibition, adsorption, stabilization and humification (Insham *et al.,* 1989; Insham, 1990; Sinsabaugh *et al.,* 1991).

As hydrolytic extracellular enzymes play a central role in organic matter remineralisation, the abiotic and biotic factors controlling enzyme activities need to be thoroughly understood (Chróst, 1992). Enzymes in general show activities with strong pH dependency because changes in hydrogen ion concentration can modify the 3D structure of the active site of an enzyme (Tipton *et al.,* 2009). Extracellular enzymes, that act outside the intracellular compartment, are mainly hydrolases (e.g., glycosidases, peptidases, esterases), the enzymes that cleave C-O and C-N bonds that link monomers (Sinsabaugh, 1994). There are extracellular enzymes that catalyze oxidative reactions, typically cleaving C-C and C-O bonds. These oxidative enzymes can be roughly divided into oxygenases and peroxidases that use molecular oxygen and hydrogen peroxide, respectively, as electron acceptors. Extracellular enzymes can be further classified according to their physical relation with the cell as

ectoenzymes and truly extracellular enzymes. Ectoenzymes are associated with viable cells and include enzymes inserted in or spanning the plasma membrane, associated with the cell wall or, in gram negative bacteria, attached to the outer membrane surface or retained within the periplasmic space by the strict exclusion limit of the outer membrane (Chróst, 1991; Skujins, 1978). Ectoenzymes ensure a close association between the hydrolytic products and its source cells, to avoid both enzyme and hydrolysis products getting easily lost to the environment (Boss *et al.,* 1996). Extracellular enzymes in its strict sense occur in free form and catalyze reactions detached from their producers (Cunha *et al.,* 2010). Extracellular enzymes are proportionally more important in the decomposition of particulate or colloidal material in the dark ocean (Baltar *et al.,* 2010; Cunha *et al.,* 2010).

In aquatic ecosystems, dissolved enzymes are also transported from the shallow permeable sediment to overlying water layers above the sediment, enhancing the extracellular enzyme activity in the water column (Cunha *et al.,* 2003; Arnosti *et al.,* 2009). These activities respond very promptly to the inputs of organic matter from sedimentation events and decreases towards the interior depths of the sediment column (Meyer-Reil 1987; Fabiano *et al.,* 1998). Sediment texture such as grain size, percentage of fineness and water content generally show significant relations with the hydrolysis rates (Hoppe, 1983). It is also important to know that the marine bacteria are the main consumers of freshly produced organic matter. Many enzymatic processes involved in the bacterial digestion of organic compounds were found to be pH sensitive in previous studies, which correlates to the rapid changes in these processes due to the continuous rise in atmospheric CO_2 concentration which causes the seawater pH decreasing at a rate unprecedented. The consequences of pH reduction on microbial physiology, organic matter cycling and marine biogeochemistry are currently under focus worldwide. The study of microbial extracellular enzymes and their determinants are thus significant in the present day context. The present study thus focuses on the extracellular hydrolytic enzyme production potential of bacterial strains isolated from the sediment domain of coastal, estuarine and mangrove ecosystems along the Kerala coast.

5.2. Materials and Methods

5.2.1. Bacterial strains

For the purpose of hydrolytic enzyme screening, the bacterial cultures were spot inoculated on nutrient agar media supplemented with specific substrates and examined for enzyme production.

5.2.2. Hydrolytic Extracellular Enzyme Production

5.2.2.1. Amylase Production

The plate assay for amylase production was carried out using the method of Mac Faddin (1980). The bacterial strains were spot inoculated on to nutrient agar medium supplemented with 0.5% soluble starch and were incubated till the colonies were clearly visible. The plates were flooded with Gram's iodine solution. The Iodine reacts with starch to give a deep blue coloration. Presence of amylase was visualized by clear zone around the culture due to starch hydrolysis. The amylase producing colonies exhibited a clear zone against a blue-black surrounding.

5.2.2.2. Protease Production

Protease production was studied by using gelatin as the substrate (Frazier, 1926). Nutrient agar medium supplemented with 2% gelatin powder was inoculated and incubated for 48 hours. Subsequently the plate was flooded with a solution of 15% Mercuric Chloride ($HgCl_2$) in 20% (Hydrochloric acid) HCl. Presence of gelatinase was visualized by clear zone around the culture due to gelatin hydrolysis.

5.2.2.3. Lipase Production

The plate assay for lipase production was carried out using the method of Anderson (1939). The strains were spot inoculated on to nutrient agar medium supplemented with 1% tributyrin and were incubated for 48 hours. Presence of lipase was visualized by a clear zone around the culture due to lipid hydrolysis.

5.2.2.4. Cellulase Production

Cellulase production was screened according to the method of Hankin and Anagnostakis (1977). The strains were spotted on to nutrient agar medium supplemented with 0.5% carboxy methyl cellulose. After incubation for 3-4 days the plates were flooded with Congo red dye (1 mg/ml) solution. It was further incubated for a period of 15 minutes at room temperature. The plates were washed several times using 1 M sodium chloride to remove the unbound excess dye. A clearance zone against a bright red background indicated the production of cellulase.

5.2.2.5. Chitinase Production

Chitin agar plates provided a convenient screening method for chitinase activity. Samples from overnight bacterial cultures were spotted onto agar plates prepared from nutrient agar containing 2% (w/w) chitin. Colloidal Chitin was prepared as reported by Lingappa and Lockwood (1962). The plates were incubated for 3 days at 30°C, and a clear zone around the stab site showed the presence of chitinase activity due to chitin hydrolysis. The plates were stored after the initial incubation for up to 4 weeks at 4°C, during which time the zone became clearer and larger.

5.2.2.6. Ligninase Production

The bacterial strains were spot inoculated on to nutrient agar medium supplemented with 0.5% tannic acid. After 4-7 days incubation the appearance of a clearance zone around the colony was taken as an indication of ligninase production (Nobles, 1964).

5.2.2.7. Phosphatase Production

The screening for phosphatase production was carried out by the method of Baird - Parker (1966). Basal nutrient agar plates containing 1 ml of 1% solution of phenolphthalein di-phosphate were spot inoculated with the bacterial culture and incubated till sufficient growth was observed. These plates were then exposed to

ammonia (NH_3) vapors by inverting it over a petri dish containing NH_3 solution. Pink coloration of cultures indicated the presence of phosphatase enzyme.

5.2.2.8. Alginase Production

The screening for alginase production was carried out by the method of Gacesa and Wusteman (1990). Nutrient agar medium supplemented with 1.5% sodium alginate were spot inoculated with bacterial strains. After incubation for 2-3 days the plates were flooded with 10% cetyl pyridinium chloride solution. The positive strains showed a clearance zone against an opaque white background after incubation for 10-30 minutes at room temperature.

5.2.2.9. Xylanase Production

Xylanolysis Basal Medium (XBM) was prepared and incorporated with 4% w/v oal spelt xylan and 1.6% w/v of agar and autoclaved. The sterile media was aseptically transferred to the sterile petri dishes and inoculated with the test organism individually. The plates were then incubated at room temperature for 48 h for bacteria and 5-7 days for fungi. The media after growth was flooded with iodine stain (0.25% w/v aqueous Iodine and Potassium Iodide) for 5 min, the stain was removed and the agar surface washed with distilled water. Xylan degradation around the colonies appeared as yellow-opaque area against a blue/reddish purple color for undegraded (Biely, 1985).

5.2.2.10. Pectinase Production

Pectin is a complex colloidal acidic polysaccharide present in the primary cell wall and middle lamella of fruits and vegetables. They are the sole polysaccharides responsible for cell cohesion. Pectinases are heterogenous group of related enzymes that catalyze breakdown of pectic substances. Purified bacterial strains were spotted on to pectin enriched agar plates which contained 5 g/L pectin. After 3 days, clear zones were visualized using 1% cetrimide solution (Beg *et al.*, 2000).

5.2.2.11. DNAse Production

This media employs the property of DNA to get precipitated on reaction with hydrochloric acid. The DNA salts readily dissolves in distilled water. The Nutrient Agar medium contains 0.2% DNA sodium salt. DNA agar plates were spot inoculated and incubated at room temperature (28 ± 2˚C) overnight. Flooded the plate with 1N HCl. Clearing around the colonies indicates the DNase activity. The HCl reacts with unchanged deoxyribonucleic acid to give a cloudy precipitate (Jeffries *et al*., 1957).

5.3. Results

5.3.1. Hydrolytic Extracellular Enzyme Production

The heterotrophic bacteria isolated during the current study exhibited potential for production of eleven different types of extracellular enzymes, with the maximum number of strains producing protease, followed by amylase and lipase (Fig.5.1).

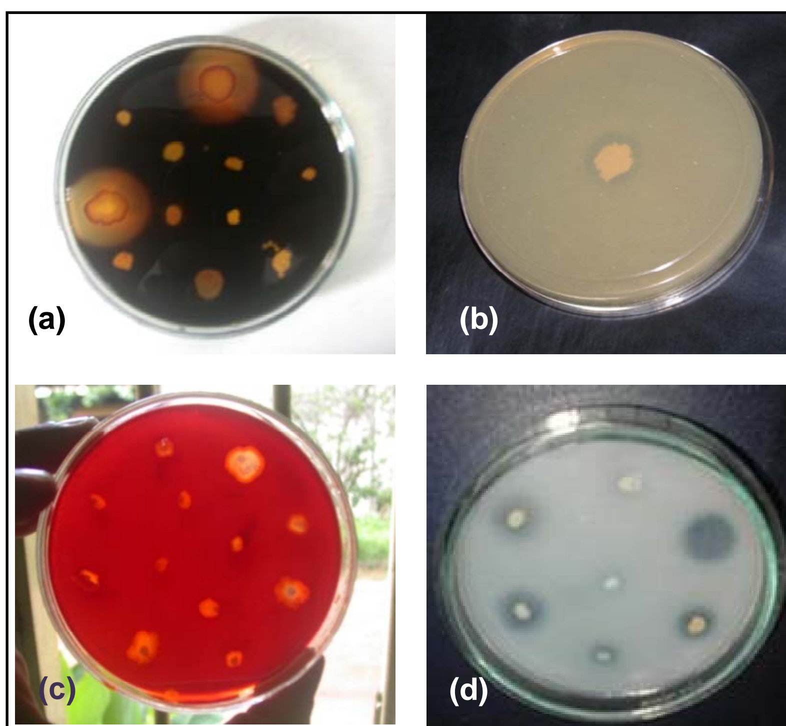


Fig. 5.1. Hydrolytic enzyme production by heterotrophic bacteria showing clearance zone around colonies a) Amylase b) Lipase c) Cellulase and d) Protease

5.3.1.1. Temporal variations in hydrolytic extracellular enzyme production

During all the three seasons of 2006-07, the majority of the isolated strains exhibited production of protease, lipase and amylase (Fig.5.2). Among the three seasons, strains isolated during post monsoon had relatively higher enzyme production potential, with 33% of the strains producing protease, followed by amylase (28%) and lipase (27%). During monsoon also, the strains exhibited comparative enzyme production potential, with 30% producing protease and amylase each and 25% producing lipase. In fact, relatively higher percentage of amylase producing strains was isolated during monsoon (30%). During pre monsoon, 30% of the isolates were protein producers, followed by amylase (27%) and lipase (25%) producing strains. A considerable percentage of the isolates also exhibited phosphatase production during pre monsoon (6%), monsoon (4%) and post monsoon (5%) seasons. Ligninase, xylanase and alginase were the least produced enzymes during all the three seasons, being produced by only 1% each of the isolates.
DNAase was also produced by only very low percentage of the isolates during the three seasons (2% each during pre monsoon and monsoon and 1% during post monsoon).

During 2007-08 also, the majority of the isolated strains exhibited production of protease, amylase and lipase (Fig.5.3). Among the three seasons, strains isolated during post monsoon had relatively higher enzyme production potential, with 34% of the strains producing protease, followed by amylase (28%) and lipase (23%). During monsoon also, the strains exhibited comparative enzyme production potential, with 32% producing protease, 31% amylase and 24% producing lipase. In fact, the relatively higher percentage of amylase producing strains was isolated during monsoon (31%). During pre monsoon, 31% of the isolates were protein producers, followed by amylase (30%) and lipase (22%) producing strains. 4% each of the isolates also exhibited phosphatase production during pre monsoon, monsoon and post monsoon seasons. Ligninase was produced by 5% of the isolates in pre monsoon, 3% in monsoon and 2% in the post monsoon season. Xylanase and alginase were the least produced enzymes during all the three seasons, being produced by only 1% each of the isolates. DNAase was not produced by any of the

isolates during pre monsoon and monsoon, while during post monsoon 2% of the isolates exhibition potential for DNAase production.

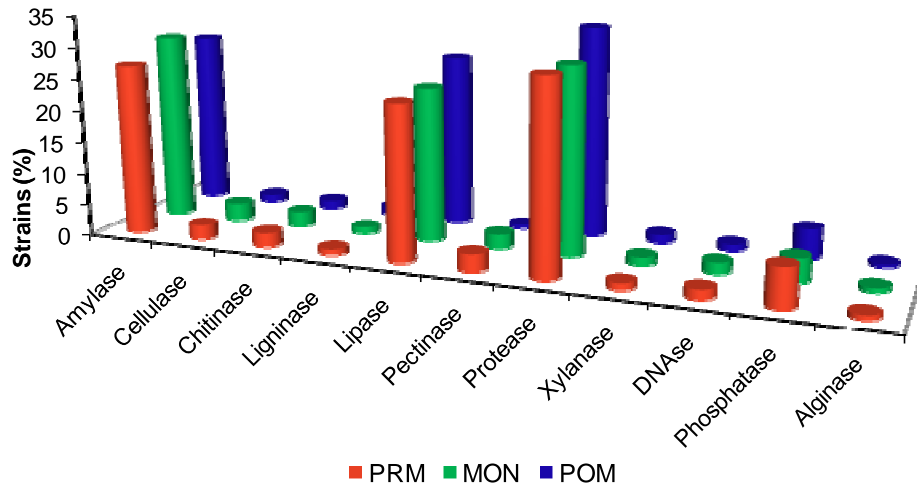


Fig.5.2. Hydrolytic enzyme production by heterotrophic bacteria isolated during the different seasons in 2006-07

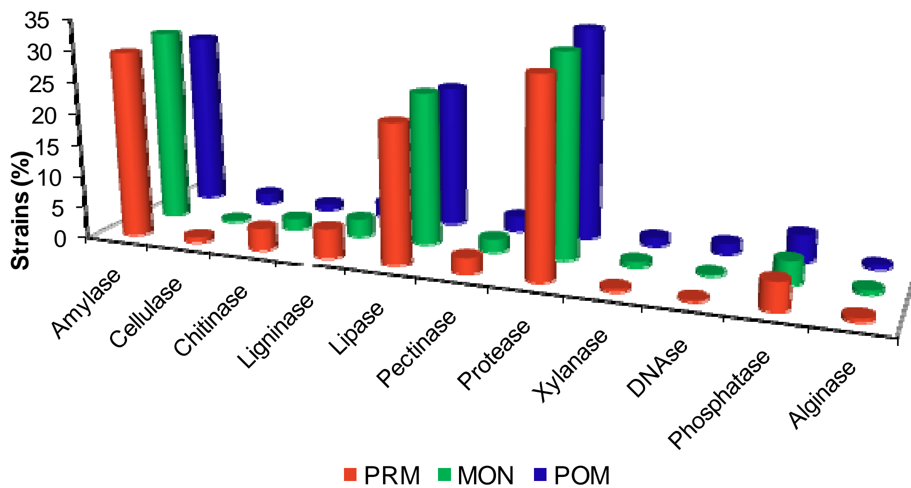


Fig.5.3. Hydrolytic enzyme production by heterotrophic bacteria isolated during the different seasons in 2007-08

5.3.2. Spatial variations in hydrolytic extracellular enzyme production

Among the three different types of ecosystems studied during 2006-07, the coastal and estuarine stations had relatively higher number of protease, amylase and lipase producing strains (Fig.5.4). During pre monsoon, the maximum number of protease (78), amylase (67) and lipase (72) producing strains were isolated from the estuarine station Fort Kochi. The mangrove stations Kadalundi and Puduvaipu had the least protease (Kadalundi - 27; Puduvaipu - 19), amylase (Kadalundi - 18; Puduvaipu - 22) and lipase (Kadalundi - 22; Puduvaipu - 18) producing strains. The mangrove station Dharmadom was slightly better with 36, 37 and 27 strains each producing protease, amylase and lipase.

In the monsoon season also, the coastal and estuarine stations harboured more number of enzyme producers than the mangrove stations. During this season, the maximum number of protease (95) and amylase (81) producing strains were isolated from the coastal station Punnapra, while the maximum number of lipase producers (75) was isolated from the estuarine station Mahe. As observed during pre monsoon, Kadalundi and Puduvaipu had the least protease (Kadalundi - 36; Puduvaipu - 25), amylase (Kadalundi - 24; Puduvaipu - 29) and lipase (Kadalundi - 29; Puduvaipu - 24) producing strains.

During the post monsoon season, the bacterial strains isolated from the estuarine stations had relatively higher enzyme production potential compared to the coastal and mangrove stations. Among them, the bacterial strains isolated from Fort Kochi had the highest protease (88) and amylase (68) production potential, while those isolated from Azheekode harboured the maximum number of lipase producing strains (72). The mangrove stations Kadalundi (protease producers - 31, amylase producers - 31, lipase producers - 25) and Puduvaipu (protease producers - 28, amylase producers - 27, lipase producers - 31) harboured the least number of protease, amylase and lipase producing strains, followed by the coastal station Kodikkal (protease - 36, amylase - 32, lipase - 28).

During 2007-08 also, the coastal and estuarine stations had relatively higher number of protease, amylase and lipase producing strains (Fig.5.5). However, unlike in 2006-07, the bacterial strains isolated from both the coastal and estuarine stations exhibited high enzyme producing potential during the post monsoon season. Among the mangrove stations, as observed in 2006-07, Kadalundi and Puduvaipu had the least protease, amylase and lipase producers, while the mangrove station Dharmadom had relatively more number of strains producing protease, amylase and lipase.

The phosphatase producing strains did not exhibit any particular trend in their distribution between the coastal, estuarine and mangrove stations. The maximum phosphatase producing strains were recorded from the coastal station Kodikkal during the pre monsoon season (2006-07: 25 strains; 2007-08: 13 strains). However, between the seasons, the bacterial strains isolated during the pre monsoon had the highest phosphatase production potential, followed by monsoon and post monsoon seasons.

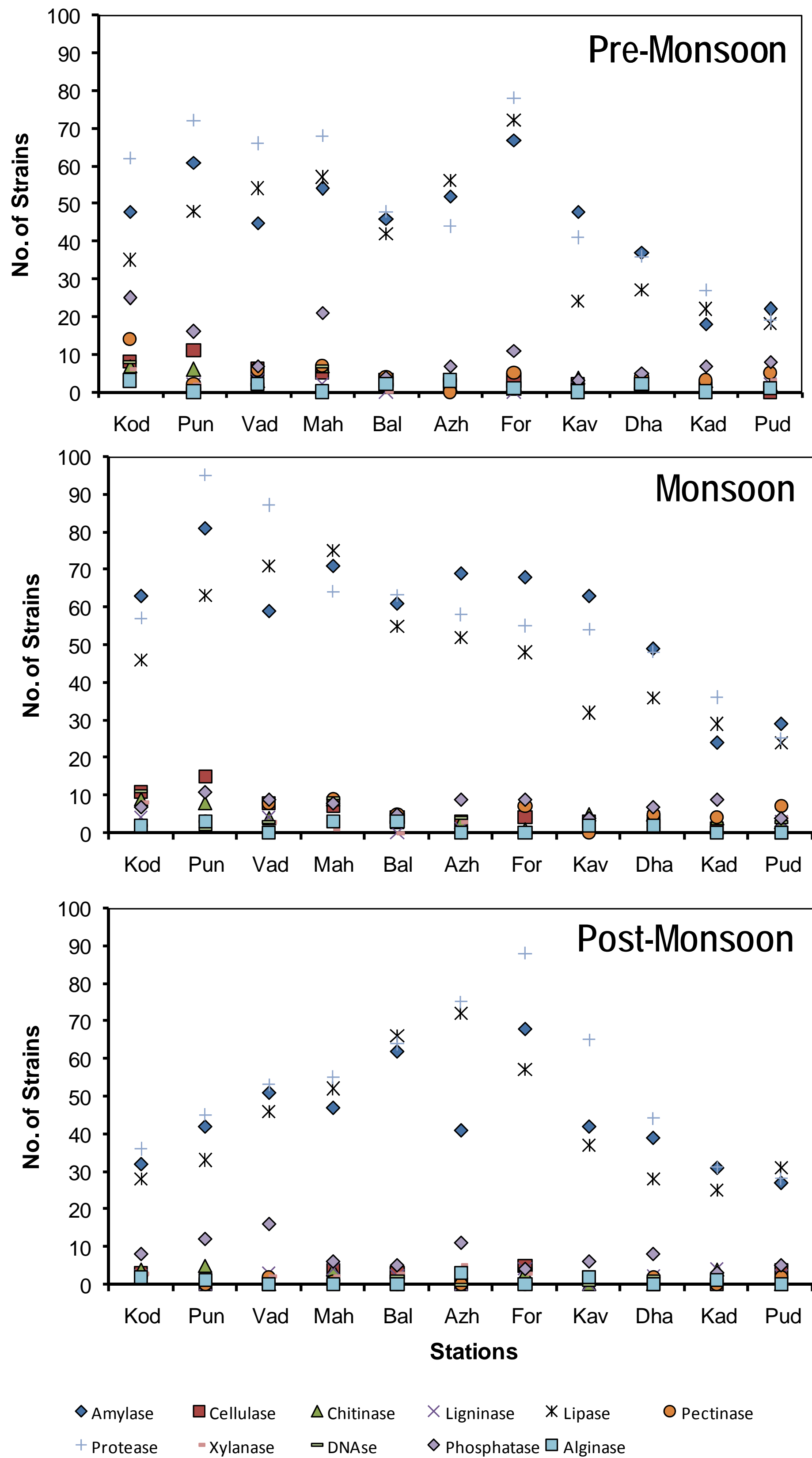


Fig.5.3. Variations in hydrolytic extracellular enzyme production by bacteria isolated from the different stations along the Kerala coast during 2006-07

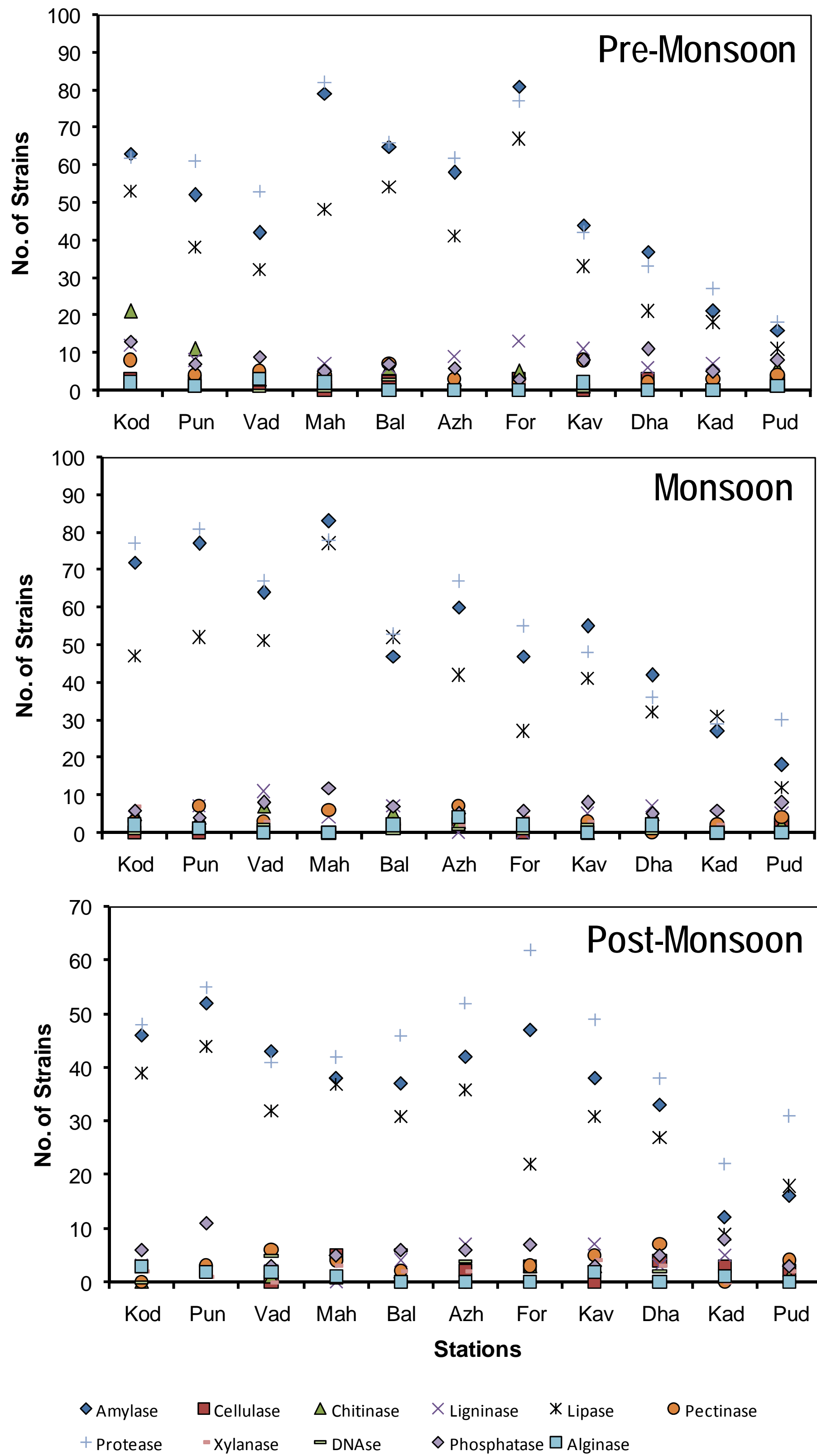


Fig.5.4. Variations in hydrolytic extracellular enzyme production by bacteria isolated from the different stations along the Kerala coast during 2007-08

5.4 Discussion

The coastal ecosystems, including estuaries, mangroves and other coastal realms, are the most productive ecosystems in the marine environment (Nair and Sujatha, 2012). The organic matter in the coastal sediments is mostly composed of carbohydrates, proteins, fats and nucleic acids. The heterotrophic bacteria in sediment quickly consume the less resistant molecules, such as the nucleic acids and many of the smaller proteins as peptides (Jedrezejczak, 1999). The sediment organic matter is basically derived from plant and animal detritus, or derived from natural and anthropogenic sources. The major anthropogenic inputs include sewage and effluent discharge from food-processing plants, pulp and paper mills and fish-farms. The increase in the concentration of complex polymeric substances in the marine sediments will result in slower bacterial action and recycling of nutrients. The extracellular enzymes produced by bacteria are thus critically important in degrading these molecules and thereby enhancing their recycling back to the water column.

Microbial degradation rates in marine sediments are dependent on molecular structures of organic substrates (Arnosti & Repeta, 1994). The production and activity of bacterial hydrolytic enzymes which facilitate microbial degradation are, in turn, dependent on the availability, distribution and concentration of organic substrates (Boetius, 1995). The enzyme production potential of the various stations would therefore reflect the trophic status of the given environment. In the present study, the high enzyme production potential of the bacterial strains isolated from the estuarine station, Fort Kochi, during pre monsoon and post monsoon seasons can be attributed to the apparent heterotrophic nature of the Cochin backwater system (Shoji *et al.,* 2008; Gupta *et al.,* 2009). The heterotrophic system, with its complex load of organic molecules, would require a battery of bacterial enzymes for effective biodegradation. The increased bacterial heterotrophic activity due to enhanced nutrient levels would result in CO_2 supersaturation and subsequent oxygen undersaturation (Garnier *et al.,* 1999; Gupta *et al.,* 2009; Abhilash *et al.*, 2012). The relatively low number of enzyme producing strains in Fort Kochi during monsoon season can be attributed to the heavy flushing as a result of the south-west monsoon.

The main components of organic matter in the marine environment are lipids, proteins, polypeptides, peptides and amino acids formed by the degeneration of dead remains of the phytoplankton, zooplankton and benthos (Billen and Fontigny, 1987). Towards degradation of these molecules, marine bacteria are known to produce protease, amylase, lipase, chitinase, cellulase, ligninase, pectinase, xylanase, nucleases etc. High proportions of bacteria isolated from marine sediments have been reported to degrade lipids and proteins (Nitkowski *et al.*, 1977). Polysaccharide degrading bacteria have also been reported in high numbers from coastal waters and sediments (Araki and Kitamikado, 1978). For heterotrophic bacteria, lipids are an important source of carbon and energy (Arts *et al.,* 1992). Therefore, majority of the marine bacteria are capable of hydrolyzing lipids, which constitute one of the most numerous physiological groups in aquatic ecosystems. Lipolytic bacteria have been shown to play a key role in the processes of modifying and transforming lipid compounds in the marine environment (Gajewski *et al.,* 1997). High lipolytic activity has been reported from marine environments like the Baltic sea sediments (Bolter and Reinheimer, 1987; Mudryk, 1998). In the Norwegian Sea, Adriatic Sea, Atlantic Ocean and Arctic Ocean, the lipolytic forms were found to constitute 60 to 100% of the total viable counts (Krstulovic and Solic, 1988). In the present study also, high proportions of protease, amylase and lipase producing bacteria were recorded. This indicates the rich organic matter composition of the coastal sediments of the study area.

Among the three different types of ecosystems, it was observed that the mangrove ecosystem had the least enzyme producing strains. This could possibly be due to the relatively low number of strains isolated from the mangrove stations. Moreover, the potential enzymatic activity of bacterial strains may not be identical to bacterial activity in natural environments. That is, since majority of the bacteria are uncultivable, the enzyme potential determined through screening experiments may not reflect the actual *in situ* conditions. In a broader sense, the in-depth analysis of the microbial loop and the inherent enzymatic activity leading to each biogeochemical cycle would be required to assess the health and status of an ecosystem. However, the results obtained in the present study could form a baseline data for further assessment of the coastal ecosystems.

CHAPTER 6

PARTIAL PURIFICATION AND CHARACTERISATION OF ALKALINE LIPASE PRODUCED BY *BACILLUS PUMILUS* AND ITS APPLICATION POTENTIAL

6.1. Introduction

Enzymes are catalytic agents produced by living cells to accomplish specific chemical reactions. They are essential for reactions associated with reproduction, growth and maturation of all living organisms.. Fundamentally, all enzymes are protein molecules with catalytic properties and potential for specific activation (Tucker 1995). Activity of an enzyme depends upon various parameters including enzyme concentration, substrate concentration, pH, temperature as well as the presence of inhibitors and co-factors.

Microorganisms are important sources of commercial enzymes. Extensive research by Takamine (1894; 1914) and Boidin and Effront, (1917) revealed that certain microorganisms produce enzymes with activities similar to amylase of malt and protease of pancreas (Underkofler *et al.*, 1957). In fact, microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to the absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1995). Industrial use of enzymes has increased manifold since the 1950s.

Marine microorganisms, being easy to isolate, maintain, identify and bioprocess, are of major interest to enzyme researchers worldwide. They are a rich source of enzymes and bioactive compounds. The vast array of microbial extracellular enzymes include proteases, lipases, amylases, peptidases, glucomylases, invertases, malt-diastases, lactases, α-galactosidases, cellulases, hemicellulases,

pectinases, chitinases, phytases, phosphatases, arylsulfatases, L-asparaginases, L-glutaminases, ureases, lactamases etc. These extracellular enzymes have numerous applications in food, dairy, pharmaceutical, agricultural, cosmetic and detergent industries. Microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Enzyme production is closely controlled in microorganisms and therefore, to improve its productivity these controls can be exploited and modified.

Proteases, carbohydrases and peroxidases have been the most reported enzymes from near-shore sediments, deep sea sediments and seawater. The remarkable among these are the detergent-resistant, alkaline serine exoprotease produced by *Vibrio alginolyticus*; BAL 31 Nuclease having endo- and exonuclease activity isolated from *Alteromonas espejiana* BAL 31, the novel metalloproteinase, almelysin produced by *Alteromonas* sp. etc. (Shibata *et al.*, 1997; Ghosh *et al.*, 2005). The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates, which is widely used in food and pharmaceutical industries. As of now, detergent industries account for the largest share of world enzyme market (Maurer, 2004). Protease and lipases are the key enzymatic constituents in detergent formulations. Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis. Therefore, lipases, especially microbial lipases have many industrial applications (Hasan *et al.*, 2006). Following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume (Liu *et al.*, 2008).

The industrial lipases are special classes of esterase enzymes that act on fats and oils, initially hydrolysing them into the substituted glycerides and fatty acids, and finally into glycerol and fatty acids (Macrae and Hammond, 1985; Ghosh *et al.*, 1996). Lipases are generally produced on lipid carbon, such as oils, fatty acids, glycerol or tweens in the presence of an organic nitrogen source. The enzyme is most

commonly purified by hydrophobic interaction chromatography, in addition to some modern approaches such as reverse micellar and aqueous two-phase systems. Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common. Lipases stand amongst the most important biocatalysts carrying out novel reactions in both aqueous and non-aqueous media. This is primarily due to their ability to utilize a wide spectrum of substrates (due to lipolytic as well as esterolytic activity) and high stability towards extremes of temperature, pH and organic solvents (Jaeger *et al.,* 1994; Jaeger and Reetz, 1998; Kazlauskas and Bornscheur, 1998; Beisson *et al.,* 2000; Gupta and Soni, 2000; Jaeger and Eggert, 2002; Gupta *et al.*, 2004).

More than 50 lipases have been identified, purified and characterized to date, which originate from natural sources such as animals, plants and microorganisms (native or genetically engineered). Among lipases of plant, animal and microbial origin, it is the microbial lipases that find immense application. This is because microbes can be easily cultivated and their lipases can catalyze a wide variety of hydrolytic and synthetic reactions. Lipases (E.C. 3.1.1.3) are produced by several microorganisms namely, bacteria, fungi, archea and eukarya (Olson *et al.,* 1994). Lipases with novel properties have been discovered from microorganisms isolated from extreme environments like Antarctic Ocean, hot springs and highly salty or sugary environments (Feller *et al.,* 1990; Elwan *et al.,* 1985; Gowland *et al.,* 1987; Lee *et al.,* 1999; Ghanem *et al.,* 2000). Lipase producing microorganisms have been reported to grow at varied pH and temperatures. The fungi usually require acidic pH for growth and lipase production (Arima *et al.,* 1772, Pokorny *et al.,* 1994), while bacteria are found to prefer neutral or alkaline pH (Gao *et al.,* 2000; Ghanem *et al.,* 2000). Psychrophilic and thermophilic microbes, as well as those having varied oxygen demand (aerobic, microaerophilic and anaerobic) are also reported to produce lipases.

A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal lipases (Peterson and Drablos, 1994). Bacterial lipases were initially isolated from *Bacillus prodigiosus*, *B. pyocyneus* and *B. fluorescens* in 1901 A.D. (Jaeger *et al.,* 1994). These bacteria, now renamed as *Serratia*

marcescens, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively, are some of the best studied lipase producers even now. Lipase producers have mostly been isolated from soil and spoiled food materials containing vegetable oils. They are also responsible for the virulence factor of many plant and animal pathogens. Bacterial lipases are mostly extracellular and are produced by submerged fermentation. *Bacillus* and related genera are one of the most important sources of novel lipases and other industrial enzymes because they have been shown to possess a lipolytic system well suited for biotechnological applications (Zock *et al.*, 1994; Dröge *et al.*, 2000). The vast diversity of bacterial lipases is enlisted in Table 6.1. Bacterial lipases play a vital role in commercial ventures.

Table 6.1. Lipases isolated from bacterial strains

Bacterial Strain	**Reference**
Achromobacter lipolyticum	Scholefield *et al.,* 1978
Acinetobacter baumannii, *A. calcoaceticus* 69-V, *A. radioresistens*	Hostacka, 2000; Haferburg and Kleber, 1983; Wang and Chen,1998
Aeromonas hydrophila MCC-2*, *A. sobria LP004*	Chuang *et al.,* 1997; Lotrakul and Dharmsthiti, 1997a
*Alcaligenes sp. strain No. 679, A. denitrificans**	Kokusho *et al.,* 1982; Odera *et al.,1986*
*Alteromonas**	Saimoku *et al.,* 1999
Anerovibrio lipolytica	Henderson, 1971
Bacillus acidocaldarius, *B. alcalophilus*, *B. atrophaeus* SB-2, *Bacillus stearothermophilus* SB-1, *B. licheniformis* SB-3, *B. circulans*, *B. pumilus*, *B. subtilis*, *Bacillus thermocatenulatus**, *B. thermoleovorans ID-1**	Manco *et al.,* 1998; Ghanem *et al.,* 2000; Bradoo *et al.,* 1999; Elwan, 1985; Mourey, 1981; Kennedy and Lennarz, 1979; Rua, 1998; Cho, 2000
Brevibacterium linens	Adamitsch and Hampel, 2000
Brochothrix thermosphacta	Papon and Talon, 1988
Burkholderia cepacia, *B. glumae*, *B. pseudomallei*	Ishii, 2001; El Khattabi, 2000; DeShazer, *et al.* 1999
Campylobacter jejuni, *C. coli*, *C. lari*	Colomina, *et al.* 1997
Chromobacterium viscosum, C. viscosum pararipoliticum	Horiuti and Imamura, 1977; Sagai *et al.* 1889

Corynebacterium sp.	Ping and Omar, 1993
Escherichia coli	Nantel *et al.,* 1978
Flavobacterium odoratum	Labuschagne *et al.,* 1997
Lactobacillus casei-subsp-casei LLG, *L. casei subsp. pseudoplantarum LE2,* *L. plantarum 2739, L. casei 2756,* *L. fermentum DT41,* *L. acidophilus A2, L. sanfranciscensis*	Lee and Lee, 1990; Lee and Lee, 1989; El-Sawah, 1995; Gobbetti *et al.,* 1996; De Angelis*, et al; 1999*
Lactococcus helveticus	Carrasco *et al.,* 1995
Leuconostoc citrovorum	Belov and Umanskii*,* 1977
Micrococcus varians CAS4	Zahran*,* 1998
Moraxella TA144*	Feller *et al.,* 1990
Mycobacterium rubrum	Lebedeva, 1977
Pediococcus pentosaceus SV61	Oestdal *et al.,* 1996
Propionibacterium arabinosum ATCC 4965*, P. shermanii* ATCC 6915*, P. acnes, Propionibacterium freudenreichii subsp. freudenreichii.*	Merilainen and Uusi-Rauva, 1976; Ingham *et al.,* 1981; Dupuis *et al.,*1993
Proteus vulgaris K80	Kim and Oh, 1998
Pseudomonas aeruginosa, *P. alcaligenes*, *P. cepacia**, *P. fragi* *, *P. fluorenscens*, *P. glumae*, *P. mendocina* 3121-1, *P. mephitica var.lipolytica*, *P. plantarii*, *P. pseudoalcaligenes*, *P.putida* 3SK, *P. stutzeri*, *P. solanacearum*, *P. tolaasii*, *Pseudomonas wisconsinensis*	Jaeger and Winkler, 1984; Holmes, 1990; Dünhaupt *et al.,* 1992; Mencher and Alford, 1967; Makhzoum *et al.,* 1995; Deveer *et al*., 1991; Bachmatova *et al.,* 1995; Bycroft *et al.,* 1990; Lin *et al.,*1996; Lee and Rhee, 1993; Piao *et al*., 1998; Kotsuka *et al.,* 1996; Baral and Fox, 1997; Charmoille, *et al.,* 1997
Selenomonas lipolytica	*Dighe et al.,* 1998
Serratia liquefaciens *S. marcescens 345*	Zou *et al*., 1996; Bashkatova and Severina, 1980
Streptococcus cremoris, S. diacetilactis, *S. faecalis, S. lactis,* *S. thermophilus*	DeMoraes and Chandan, 1982; Kamaly *et al.,* 1990; Chander *et al*., 1979; Belov and Umanskii,1977
Streptomyces exfoliates M11,* *S. cinnamomeus,*	Servin-Gonzalez *et al*., 1997; Sommer *et al*., 1997;

S. parvulus, S. clavuligerus, S. coelicolor, S. rimosus	El-Shirbiny and Ghaly, 1992; Large *et al.,* 1999
Staphylococcus aureus, S. epidermidis, S. haemolyticus, S. hyicus, S. carnosus*, S.warneri and S. xylosus*	Bisignano 1980; Farrell *et al.*, 1993 Oh *et al.*, 1999; Goetz *et al.*, 1985, Voit *et al.* 1991; Talon *et al.* 1996
Thermoactinomyces vulgaris	Elwan *et al.,* 1978
Thermus sp.	Silva *et al.,* 1991
Thermosyntropha lipolytica gen. nov., sp. nov.	Svetlitshnyi *et al.,* 1996
Vibrio cholerae	El Ogierman *et al.,* 1997
Xanthomonas campestris pathovar *sesami*	Sheela *et al.,* 1996
Yersinia	Kuznetsov and Bagryantsev, 1992

Most of the bacterial lipases reported so far are constitutive and are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable. Among bacteria, *Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Aeromonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp. and *Chromobacterium* sp. have been mostly exploited for the production of lipases. Also, the marine *Vibrio* sp. VB-5 has been reported to produce a lipase capable of hydrolyzing fish oil containing n-3 polyunsaturated fatty acid (PUFA). It is believed to catalyse the esterification reaction with n-3 PUFA (Chandrasekaran and Kumar, 2002).

The use of lipases in industrial applications has grown rapidly in recent years and is likely to markedly expand further in the coming years. Lipases find various uses in production of fatty acids (Linko *et al.,* 1990; Marangoni, 1994), biosurfactants (Chopineau *et al.,* 1988), aroma and flavor compounds (Gandhi *et al., 1995),* lubricant and solvent esters (Linko *et al.,* 1994), polyesters (Linko *et al.,* 1995b), and biomodified fats (Marangoni, 1994; West, 1988). Lipases are also widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Kazlauskas and Bomscheuer, 1998). Lipase can be used to accelerate the degradation of fatty waste (Masse *et al.,* 2001a; Masse *et al.,* 2001b) and polyurethane (Takamoto *et al.,* 2001). Most of the industrial microbial lipases are derived from fungi and bacteria. There is a renewed interest in the development of more industrial applications of

lipases. For each application, the lipase selection is based on specific characteristics including substrate, positional and stereoisomer specificity as well as temperature and pH stability (Yamaguchi and Mase, 1991). The use of lipases for the modification of the positional distribution of fatty acids in butter fat triacylglycerols was reported (Safari and Kennasha, 1994). It was shown that the interchange of palmitic or myristic acid with oleic acid at the sn-2-position of the glycerol backbone can suppress the cholesterol-raising potential of milk fat (Hayes *et al.,* 1991). Lipases from *Pseudomonas fluorescens* (Kalo *et al., 1989), Aspergillus niger* (Kalo *et al.,* 1988b), *Candida cylindracea* (Kalo *et al.,* 1988a), and *Mucor mielIei* (Kalo *et al.,* 1988b). Among several commercial enzymes, lipase from *Rhizopus niveus* showed an interesting potential for the production of inter-esterified butter fat with an increased proportion of oleic acid at the sn-2 position (Safari and Kennasha, 1994). *R. japonicus* lipase has been used to produce hard butter suitable for chocolate manufacture by inter-esterification of palm oil with methy stearate (Matsuo *et al.,* 1981). The importance of thermostable lipases for different applications has been growing rapidly. Most of the studies realised so far have been carried out with mesophilic producers. Many lipases from mesophiles are stable at elevated temperatures (Sugihara *et al.,* 1991). Thermostable lipolytic enzyme has been used for the synthesis of biopolymers, biodiesel, pharmaceuticals, agrochemicals, cosmetics and flavouring agents (Haki and Rakshit, 2003).

The lipase catalysed transesterification in organic solvents is an emerging industrial application such as production of cocoa butter equivalent, human milk fat substitute "Betapol", pharmaceutically important polyunsaturated fatty acids (PUFA) and production of biodiesel from vegetable oils (Jaeger and Reetz, 1998; Nakajima *et al., 2000). Mucor miehei* (IM 20) and *Candida antarctica* (SP 382) lipases were used for esterification of free fatty acids in the absence of organic solvent or transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols (Akoh, 1993). Immobilized *M miehei* lipase in organic solvent catalyzed the reactions of enzymatic inter-esterification for production of vegetable oils such as corn oil, sunflower oil, peanut oil, olive oil and soybean oil containing omega-3 polyunsaturated fatty acids (Li and Ward, 1993).

The scope for the application of lipases in the oleochemical industry is enormous. Fats and oils are produced worldwide at a level of approximately 60 million ton/annum and a substantial part of this (more than 2 million ton/annum) is utilized in high energy consuming processes such as hydrolysis, glycerolysis and alcoholysis. The saving of energy and minimization of thermal degradation are probably the major attractions in replacing the current chemical technologies with biological ones. Miyoshi Oil & Fat Co., Japan, reported the commercial use of *Candida cylindracea* lipase in the production of soaps (McNeill *et al.,* 1991). The company claimed that the enzymic method yielded a superior product and was cheaper overall than the conventional Colgate-Emery process. Lipases are used in the textile industry to assist in the removal of lubricants, in order to provide the fabric with greater absorbency for improved levelness in dyeing. Commercial preparations used for the designing of denim and other cotton fabrics, contains both alpha amylase and lipase enzymes (Cortez, 2000).

Lipases have been used for addition in food to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known flavour and fragrance compounds (Macedo *et al.,* 2003). Lipases are used in production of leaner meat such as in fish. The fat is removed during the processing of the fish meat by adding lipases and this procedure is called biolipolysis. The lipases also play an important role in the fermentative steps of sausage manufacture and to determine changes in long-chain fatty acid liberated during ripening. Earlier, Iipases of different microbial origin have been used for refining rice, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine (Seitz, 1974).

Lipases are also important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or increasing levels can indicate certain infection or disease. Lipases are used in the enzymatic determination of serum triglycerides to generate glycerol, which is subsequently determined by enzyme-linked colorimetric reactions. The level of lipases in blood serum can be used as a diagnostic tool for detecting conditions such as acute pancreatitis and pancreatic injury (Lott and Lu, 1991).

Unichem International (Spain) has launched the production of isopropyl myristate, isopropyl palmitate and 2- ethylhexylpalmitate for use as an emoIlient in personal care products such as skin and suntan creams, bath oils etc. Immobilized *Rhizomucor meihei* lipase was used as a biocatalyst. Retinoids (Vitamin A and derivatives) are of great commercial potential in cosmetics and pharmaceuticals such as skin care products. Water-soluble retinol derivatives were prepared by catalytic reaction of immobilized lipase (Maugard *et al.,* 2002). Lipases have also been used in hair waving preparation (Saphir, 1967), as a component of topical antiobese creams (August, 1972) and in oral administration (Smythe, 1951).

The quality of black tea is dependent largely on the dehydration, mechanical breaking and enzymatic fermentation to which tea shoots are subjected. During manufacture of black tea, enzymatic breakdown of membrane lipids initiate the formation of volatile products with characteristic flavour properties, emphasize the importance of lipid in flavour development. Lipase produced by *Rhizomucor miehei* enhanced the level of polyunsaturated fatty acid observed by reduction in total lipid content (Latha and Ramarethinam, 1999).

Lipases isolated from the wax moth *(Galleria mellonella)* were found to have a bacteriocidal action on *Mycobacterium tuberculosis* (MBT) H37Rv. Lipases may be used as digestive aids (Gerhartz, 1990). Lipases are the activators of Tumor Necrosis Factor and therefore can be used in the treatment of malignant tumors (Kato *et al.,* 1989). Lipases have earlier been used *as* therapeutics in the treatment of gastrointestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies, etc. (Mauvemay *et al., 1970).* Lipase from *Candida rugosa* has been used to synthesize Iovastatin, a drug that lower serum cholesterol level. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride, a widely used coronary vasodilator, was carried out with *S. micescens* lipase (Matsumae *et al.*, 1993).

Lipase enzymes can remove fats and grease from skins and hides, particularly those with a moderate fat content. Both alkaline stable and acid active lipases are used in skin and hide degreasing. The enzyme loosens and removes the hair on the

skins, which can then be filtered off. The end product is of a higher quality when compared to leather manufactured using traditional methods. *Rhizopus nodosus* lipase was used for the degreasing of suede clothing leathers from wooled sheep skins (Muthukumaran and Dhar, 1982).

Lipases are utilized in activated sludge and other aerobic waste processes, where thin layers of fats must be continuously removed from the surface of aerated tanks to permit oxygen transport. This skimmed fat-rich liquid is digested with lipases (Bailey and Ollis, 1986) such as that from *Candida rugosa.* Both *P. aeruginosa* LP602 cells and the lipase were shown to be usable for lipid-rich wastewater treatment (Dharmsthiti and Kuhasuntisuk, 1998). Fats in wastewater treatment plants that contains mainly triglycerides is hydrolysed by immobilized lipase (Tschocke, 1990). Bacterial lipases are involved in solution of such environmental problems as the breakdown of fats in domestic sewage and anaerobic digesters (Godfrey and Reichelt, 1983). The lipase from *Pseudomonas cepacia* was found to be effective in catalysing the methanolysis and ethanolysis of grease (Hsu *et al.,* 2002). A mixture of industrial cellulase, protease, and lipase, in equal proportion by weight, reduced total suspended solids (TSS) by 30-50% and improved settling of solids in sludge. An increase in solid reduction was observed with increasing enzyme concentration (Parmar *et al*., 2001).

Monitoring of soil microbial lipase activity is a valuable indicator of diesel oil biodegradation in freshly contaminated, unfertilized and fertilized soils (Margesin *et al.,* 1999). Fungal species can be used to degrade oil spills in the coastal environment, which may enhance eco-restoration as well as in the enzymatic oil processing in industries (Gopinath *et al., 1998).*

The limited (and fast diminishing) resources of fossil fuels, increasing prices of crude oil, and environmental concerns have been the diverse reasons for exploring the use of vegetable oils as alternative fuels (Shah *et al.,* 2004). Immobilized *Pseudomonas cepacia* lipase was used for the trans-esterification of soybean oil with methanol and ethanol (Noureddini *et al.,* 2005). Fatty acid ethyl esters have also been prepared from castor oil using n-hexane as solvent and two commercial lipases,

Novozyme 435 and Upozyme IM, as catalysts (Oliveira *et al.,* 2004). Novozyme 435 have also been used to catalyse the transesterification of crude soybean oils for biodiesel production in a solvent-free medium (Du *et al., 2004).* Simple alk-yl ester derivatives of restaurant grease were prepared using immobilized lipases from *Thermomyces lanuginose* and *Candida antarctica,* as biocatalysts (Hsu *et al.,* 2002). Fatty acids esters were produced from two Nigerian lauric oils, palm kernel oil and coconut oil, by trans-esterification of the oils with different alcohols using PS30 lipase as a catalyst.

Research on microbial lipases in India date back to late seventies when a few reports on screening and production of lipase from a few fungi and bacteria appeared (Akhtar *et al*., 1980; Chander *et al*., 1980). The initial emphasis on screening exercises was followed by process optimization of maximum lipase production. Physico-chemical conditions of lipase production by *Mucor racemosus, Aspergillus wenlii,* and *Penicillium chlysogenum* was reported (Akhtar *et al.,* 1980; Chander *et al.,* 1980). Lipolytic activity of thermophilic fungi of paddy straw compost was reported (Satayanarayan and Johri, 1981). *Aspergillus niger, A. flavus, A. fumigatus* and *Penicillium glaucum* were reported as the potential lipase producers isolated from the kernels of chironji and walnut (Saxena *et al.,* 1999). Large-scale process optimization for lipase production was reported for *Aspergillus tereus, Aspergillus carneus* and *Bacillus stearothermophilus* (Yadav *et al.,* 1998a). Extracellular microbial lipases were utilized for trans-esterification reactions for producing valuable transformed edible oils which cannot be obtained by chemical inter-esterification methods (Chakrabarty *et aI.,* 1987). Lipases from *Humicola lanuginosa* and *Yarrowia lipolytica* have also been reported for the synthesis of geranyl esters (Chand and Kaur, 1998). An extracellular lipase isolated from the conidia of *Neurospora crassa,* had an apparent molecular weight of 54 kDa and 27 kDa, suggesting thereby the presence of two identical subunits (Kundu *et al.*, 1987). Extensive work on various aspects of lipase from production and purification to characterization and industrial applications has been carried out on various fungi and bacteria (Ghosh *et al.,* 1996; Yadav *et al.,* 1998a). Novel thermostable and alkaline lipases from *Aspergillus terreus* and *Aspergillus carneus* are being developed for the production of biosurfactants, glycerides and pharmaceutically important compounds.

Lipase from a strain of *Bacillus stearothermophilus* shows remarkable activity even at 100°C. Besides this, a rapid zymogram for lipase activity in polyacrylamide gels was developed (Yadav *et al.,* 1998b). The ability of lipases to show increased stability and selectivity in organic solvents has been exploited (Parmar *et al.,* 1992). Biotransformations on polyacetoxy arylmethyl ketones, benzylphenylketone peracetates, esters of polyacetoxy aromatic acids, and peracetylated benzopyranones, using commercial lipases are reported (Parmar *et al.,* 1992). The enantioselective behaviour of microbial lipases for the resolution of racemic drugs (Qazi, 1997), lipase-catalysed ester interchanges for the modification of selected Indian vegetable oils into cocoa butter substitutes and high oleic oils (Sridhar *et al.,* 1991) and enhancement of enzyme activity in aqueous-organic solvent mixtures (Gupta, 1992) were reported.

Lipases are among the highly focused enzymes today due to its enormous industrial applications. As of now, most of the lipase formulations marketed in India are imported and therefore are not economically reasonable. Therefore development of indigenous lipases is necessary. India has rich and diverse wealth of unexplored flora and fauna. This is more so in the case of marine environment. Being a tropical country, with a long coastline and blessed with an EEZ of 2.02 m Sq Km, we have enormous potential in isolation of lipolytic bacteria. In view of this, the bacteria isolated from the sediments of the mostly unexplored coastline along Kerala, situated in South-west coast of India, was screened for lipase-producing bacterial strains. During the current study, 58.98% of the total isolated bacteria were found to have potential for lipase production. Among these, 32.06% belonged to the genus *Bacillus.* Majority of the strains belonging to the genus *Bacillus* are considered as Generally Regarded as Safe (GRAS) group of organisms, making them the preferred choice for microbial resource exploitation studies. Lipase production by one of the most potent *Bacillus* strain has been studied in detail.

6.2. Materials and Methods

From the 455 lipase-producing strains determined by primary screening, the 16 most potent strains were taken up for secondary screening.

6.2.1. Secondary Screening of Lipase producers

6.2.1.1. Spirit Blue Agar Plate Assay

Spirit Blue Agar (Starr, 1941) was employed for the detection and enumeration of lipolytic microorganisms. It is a basal medium to which lipid substrate is added for the detection, enumeration and study of lipolytic microorganisms. If lipase is secreted by the bacterium, the lipids in the media is digested, which causes the normally opaque and light blue color media, to turn yellow halos around colonies. The dye spirit blue changes color due to the change in pH induced due to lipase production.

6.2.1.2. Rhodamine-B Plate Assay for true lipase production

The screening for true lipase production was carried out using Rhodamine B-olive oil agar plate method according to Kouker & Jaeger (1986). The Rhodamine agar medium was prepared in distilled water, autoclaved, and cooled to 60°C. The cooled medium was added with 3 % of olive oil that was previously sterilized. Cultures from the nutrient agar slants were streaked onto the Rhodamine B-olive oil agar plate and incubated at 45°C for 2 days. The fluorescence dye in the Rhodamine B agar plate assay indicates the zone of lipolysis as an orange fluorescence under UV light at 350 nm (Kouker and Jaeger, 1987).

6.2.1.3 . Lipase production in liquid broth (submerged fermentation)

Potent strains after secondary screening, were grown in ZoBell's marine broth supplemented with four different lipid sources, namely, Tributyrin, Tween 20, olive oil and coconut oil at 1% (v/v) level. The lipolytic activity of the culture supernatant was estimated after 48 hrs of incubation.

6.2.1.4. Lipase Assay

The lipase activity was estimated spectrophotometrically by employing the para-nitrophenyl palmitate (*p*NPP) assay (Winkler and Stuckmann, 1979) as modified by Gupta *et al.,* 2002. This assay protocol forms the colorimetric estimation of para-nitrophenol (pNP) released as a result of enzymatic hydrolysis of *p*NPP at

410 nm and is widely utilized by researchers for estimating the esterolytic activity of both lipases and esterases (Gupta *et al.,* 2002). *Para*-NPP (30 mg) dissolved in 10ml solvent (isopropanol) and 90 ml phosphate buffer (pH 7.0) in conjunction with sodium deoxycholate, and gum arabic, making a final concentration of 790 µM pNPP. To 2.4 ml of the above substrate solution, 0.1ml of the enzyme was added and the reaction performed at the 37° C for 10 minutes. 50 µl of Triton X-100 was added to the reaction mixture so as to disperse the fatty acids released due to the hydrolysis of pNPP (Gupta *et al.*, 2002). Following the enzyme reaction, the amount of product para-nitrophenol (*pNP*) released was estimated spectrophotometrically by measuring the optical density at 410nm. One unit of enzyme activity is defined as the amount of enzyme liberating 1 µmol of *p*-nitrophenol per minute.

6.2.2. Phenotypic and molecular identification of the selected strain

6.2.2.1. Morphological and Phenotypic characterization

The morphological and phenotypic analysis was carried out on the selected isolate. A series of biochemical tests were carried out for the identification of the selected strains, which included nitrate reduction, anaerobic growth, gas production from glucose, Voges-Proskauer (VP), growth at different NaCl concentrations, temperature and pH ranges, degradation of starch, casein, urea, growth in tweens, gelatin, chitin, acid production from arabinose, mannitol, xylose, glucose, lactose, citrate utilization and production of DNAse. In addition the production of extracellular enzymes namely caseinase, chitinase, protease, alkaline phosphatase, and gelatinase was also verified (Farrows *et al.,* 1994).

6.2.2.2. Molecular Characterization

(i) 16S rRNA Analysis

Total genomic DNA of the selected isolate was extracted by the phenol-chloroform method modified from Ausbel *et al.,* (1987). The small subunit 16S rRNA gene was amplified using the two primers 16S1 (5'-GAGTTTG ATCCTGGCTCA-3') and 16S2 (5'-ACGGCTAC CTTGTTACGACTT-3'), which are complementary to the conserved regions at the 5'- and 3' ends of the 16S rRNA gene (Lane, 1991). Genomic DNA was extracted from bacterial isolates by standard

method of Sambrook *et al.*, (1989). The 16S rRNA genes were amplified using universal bacterial primers (Rainey *et al.*, 1996). Amplification was performed in Veriti Thermal Cycler (Applied Biosystems, USA) in 25 µl reaction volume containing, 20–50 ng of DNA template and 1X GT PCR master mix. The final volume of the PCR mixture was adjusted by nuclease-free PCR grade water. A reagent blank containing all components of the reaction mixture except template DNA, was included in every PCR procedure. The thermal cycling include an initial denaturation at 95°C for 5min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis and purified using the QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's suggested protocol. Sequencing reaction of purified PCR product was performed using Big Dye Terminator Kit according to manufacturer's instructions (Applied Biosystems) and the sequences were determined using an ABI-3500 genetic analyzer (Applied Biosystems Inc., Foster City, USA). Nearly complete sequences of 16S rRNA genes were obtained using the internal primers (Jang *et al.*, 2005).

(ii) Molecular phylogeny analysis

The bacteria was identified based on the 16S rRNA gene sequence of CU/Sed/Lip/AB-11 generated with bases aligned with 16S rRNA sequences of other closely related *Bacillus* species retrieved from the EZTAXON database. A sequence similarity search was done using GenBank BLASTN (Altschul *et al.*, 1997). Sequences of closely related taxa were retrieved, aligned using the multiple sequence alignment program ClustalW (Larkin *et al.,* 2007) and the aligned sequences were then checked for gaps manually corrected. Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985). For neighbor joining analysis (Saitou and Nei, 1987) the distances between the sequences were calculated using Kimura two-parameter model (Kimura, 1980). These analyses of the sequences were conducted using MEGA version 4 software.

6.2.3. Optimization of Conditions for the enzyme production

6.2.3.1. Inoculum preparation

For the preparation of inoculum, a loop full of cells from the freshly grown culture (Nutrient Agar slant) was transferred to a 5 ml Nutrient Broth (NB) and incubated at 28 ± 2°C on a rotary shaker. After 18 hrs of incubation this preculture was again transferred to a 50 ml NB in a 250 ml conical flask and incubated at room temperature (RT) on a rotary shaker at 180 rpm. Later after 18 hrs of incubation cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C. The settled cell pellet was resuspended in 10ml of 0.8% physiological saline (5 x 10^8 cfu /ml) and used to inoculate the production media.

(ii) Enzyme Production in Mineral Medium

The use of a minimal media would help to know the exact nutritional requirements of the organisms studied. Minimal media was used as the basal media for evaluating the efficiency of the strains for their ability to use various lipid substrates as the sole source of carbon (Appendix I).

6.2.3.2. Effect of various lipid substrates on the lipase production

The selected isolate was grown with different lipid substrates like Tweens (20, 40, 60 and 80), Tributyrin, Castor Oil, Olive oil, Coconut Oil, Groundnut Oil, Mustard Oil and Sunflower Oil in the minimal media and evaluated for their lipase activity. Lipid substrate was added to mineral medium at 1% (v/v) level and the lipolytic activity of the culture supernatant was estimated after 24 hours of incubation.

6.2.3.3. Effect of the concentration of Olive oil on the lipase production

Olive oil was supplemented in the mineral medium at various concentrations ranging from 0.5 to 5% v/v and inoculated with the. The lipolytic activity of the culture supernatant was estimated after 24 hours of incubation.

6.2.3.4. Effect of temperature on lipase production

The olive oil medium was inoculated with the culture and incubated at different temperatures ranging from 15°C to 70°C and the respective organisms was inoculated to check the optimum pH and its effect on lipase production. The lipase activity in the culture supernatant was determined as described earlier after 24 hours of incubation.

6.2.3.5. Effect of time period of incubation on lipase production

The time course of lipase production was studied in the olive oil medium in shake flasks incubated for 72 hours. Enzyme production was monitored at different time intervals (0, 1, 2, 6, 9, 12, 18, 24, 36, 60 and 72 hr). A 5% inoculum was added to 100 ml of medium, in 250 ml Erlenmeyer flasks and incubated at 150 rpm on a rotary shaker, at 28°C. Samples were removed periodically at various intervals and lipase activity in the culture supernatant was determined as described earlier.

6.2.3.6. Effect of pH on lipase production

The olive oil medium was adjusted to different pH ranges from 4 to 12 using HCl and NaOH. The culture was inoculated and incubated to check the optimum pH and its effect on lipase production. The lipase activity in the culture supernatant was determined after 24 hours as described earlier.

6.2.3.7. Effect of agitation speed on lipase production

The effect of aeration on the growth and protease production was determined by growing the strains in the olive oil medium and incubating them at different rpm (0, 50,100,150, 200 and 250) in a rotary shaker. Enzyme production was determined after 24 hours of incubation.

6.2.3.8. Effect of Carbon source on lipase production

Effect of various carbon sources on enzyme production was determined by inoculating the cultures in olive oil medium containing different sources of carbon. Fructose, glucose, glycerol, lactose, maltose, mannitol, mannose, sucrose and xylose

were supplemented to the mineral media at 0.5 % concentrations and the resultant enzyme productions were determined after incubation of 24 hour at 28°C.

6.2.3.9. Effect of Nitrogen source on lipase production

Effect of nitrogen sources on enzyme production was determined by inoculating the cultures in olive oil medium containing various nitrogen sources at 1% concentration. The nitrogen sources used were beef extract, casein, tryptone, peptone, yeast extract, KNO_3, $(NH_4)_2SO_4$, NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$ and urea. Enzyme production was determined after incubation of 24 hour at 28° C.

6.2.3.10. Effect of Glucose concentrations on lipase production

The effect of glucose concentration was estimated by supplementing the olive oil mineral medium broth with various concentrations of glucose ranging from 0.2 to 2 %. Enzyme production was determined after incubation of 24 hour at 28° C.

6.2.3.11. Effect of concentration of Peptone on lipase production

The effect of peptone concentration on the lipase production was estimated by supplementing the olive oil mineral medium broth with various concentrations of peptone ranging from 0.2 to 2 %. Enzyme production was determined after incubation of 24 hour at 28° C.

6.2.3.12. Effect of NaCl concentration on lipase production

The effect of NaCl concentration on growth and enzyme production was studied by inoculating the organisms in the olive oil medium having varying NaCl concentrations (0, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%). The cultures were incubated at 28°C for 24 hour and the enzyme production was measured.

6.2.4. Enzyme and Protein Assay

6.2.4.1. Assay of lipase activity

Lipase was assayed by the *p*NPP method as described in the earlier section 6.2.1.4.

6.2.4.2. Assay for Protein estimation

Protein content was estimated according to the method of Lowry *et al.*, (1951), using Bovine Serum Albumin (BSA) as the standard. OD was measured at 750 nm and expressed in milligram per milliliter (mg/ml).

6.2.4.3. Specific Activity

Specific activity of the sample was calculated by dividing the enzyme units with the protein content and was expressed as U/mg protein#

6.2.4.4. Relative Activity

Relative activity is the percentage enzyme activity of the sample with respect to the sample for which maximum activity was obtained.

6.2.4.5. Residual Activity

Residual activity is the enzyme activity of the sample with respect to the original enzyme activity of the control sample, which is expressed in percentage.

6.2.5. Partial Purification of enzyme

6.2.5.1. Ammonium Sulphate Precipitation

Purification of the alkaline proteases from the selected culture was carried out by ammonium sulphate precipitation followed by the dialysis. To the chilled cell free culture fluid, solid ammonium sulphate was added with gentle stirring, up to 80% saturation and kept overnight at 4°C. Ammonium sulphate required to precipitate lipase enzyme was optimized by the addition of various concentrations of ammonium sulfate (20%, 40%, 60% and 80% saturation) to the crude extract. The precipitate was collected by centrifugation and was dissolved in a minimum quantity of 0.1 M Tris HCl buffer (pH 8). The solution was dialyzed overnight against the same buffer at 4°C. This dialyzed enzyme was used for further studies.

6.2.5.2. Sephadex G-100 gel exclusion column chromatography

The concentrated solution of approximately 0.2ml was applied to a Sephadex G-100 gel filtration column (2.6×30 cm) equilibrated with Tris–HCl buffer (50 mM, pH 8.0) and eluted with the same buffer. The eluants from the chromatographic column were analyzed for total protein (A_{280}) and lipase activity. The fractions showing the highest lipase activity were pooled and assayed for protein content. The specific activity of the purified enzyme was compared with that of the crude enzyme, and the purification factor was calculated.

6.2.5.3. Determination of Molecular weight of the enzyme (PAGE) and activity staining for Detection of Lipase Activity

The active fractions collected after gel exclusion chromatography were concentrated and subjected to electrophoresis by Native PAGE and SDS-PAGE in a 10% polyacrylamide gel according to the method of Laemmlli, 1970. Molecular weight of the enzyme was determined by using standard molecular weight markers. Detection of the purified lipase was carried out according to Diaz *et al.*, (1999) using 4 Methyl umbelliferyl butyrate (MUF-butyrate) (Sigma) a fluorogenic substrate. After the run, the SDS- PAGE gel was soaked for 30 min in 2.5% TritonX-l00 at room temperature, briefly washed in 50mM Tris buffer, pH 8, and submerged in a solution of 100uM methyl umbelliferyl butyrate (diluted with 50 mM Tris buffer) and incubated for a short period at room temperature. Activity bands were observed under UV illumination. After the zymogram analysis, the gel was stained with Coomassie Brilliant Blue R-250 for visualizing protein bands.

6.2.5.4. Properties of the purified lipase

6.2.5.4.1. Effect of pH on the activity and stability of enzyme

Optimum pH for maximal activity of the purified enzyme was determined by conducting enzyme assay at various levels of pH in the range of 2-13. The enzyme solution used was 0.2 ml of diluted sample and the substrate pNPP was prepared in the respective buffer of each pH. The buffer systems used were, Citrate-Phosphate buffer (pH 3 to 6), Tris-HCl buffer (pH 7.0 and 8.0), Carbonate-bicarbonate buffer (pH 9 and 10). Enzyme activity and Relative activity were calculated as described

earlier. Stability of the purified enzyme over a range of pH was determined by measuring the residual activity at pH 7.0 after incubating the enzyme in different buffer systems of pH 4-12 for varying periods of incubation.

6.2.5.4.2. Effect of temperature on the activity and stability of enzyme

Effect of temperature on enzyme activity was determined by incubating the reaction mixture at different temperatures ranging from 20°C to 80°C and measuring the enzyme activity. To study the effect of temperature on enzyme stability, the dialyzed enzyme was pre incubated at different temperatures ranging from 20°C to 85°C for one hour and the residual activity was assayed at the optimum temperature of enzyme activity.

6.2.5.4.3. Effect of surfactants on the stability of enzyme

Effect of various surfactants (SDS, CTAB, Triton X 100 and Tween 20 at 0.1, 0.5 and 1% each) on the enzyme stability was determined by pre-incubating with the above-mentioned surfactants for 1 hour and then assayed under standard conditions.

6.3. Results

6.3.1. Secondary screening for lipase production

In the Spirit blue agar plate assay, all the tested 16 strains indicated positive results. This confirmed the lipase-producing potential of the tested strains.

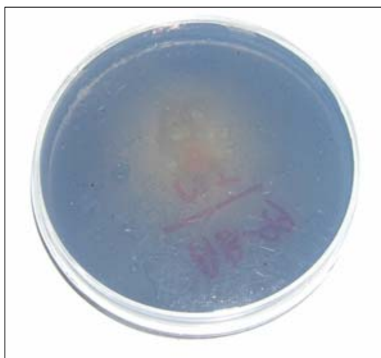


Fig. 6.1. Plate assay showing positive growth in Spirit blue agar for the Bacillus strain AB-11

In the Rhodamine B agar test also, all the 16 strains indicated positive results, confirming their potential for true lipase production.

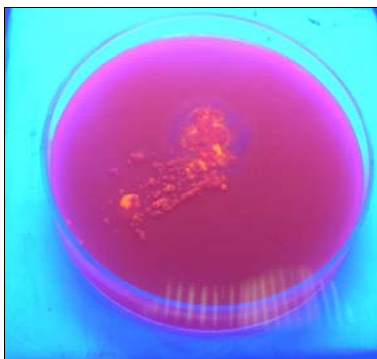


Fig. 6.2. Plate assay showing positive growth in Rhodamine B agar for the Bacillus strain AB-11

Upon testing of lipolytic activity, strain AB-11 was found to have maximum lipase production for all the four different types of substrate used (Fig.6.3).

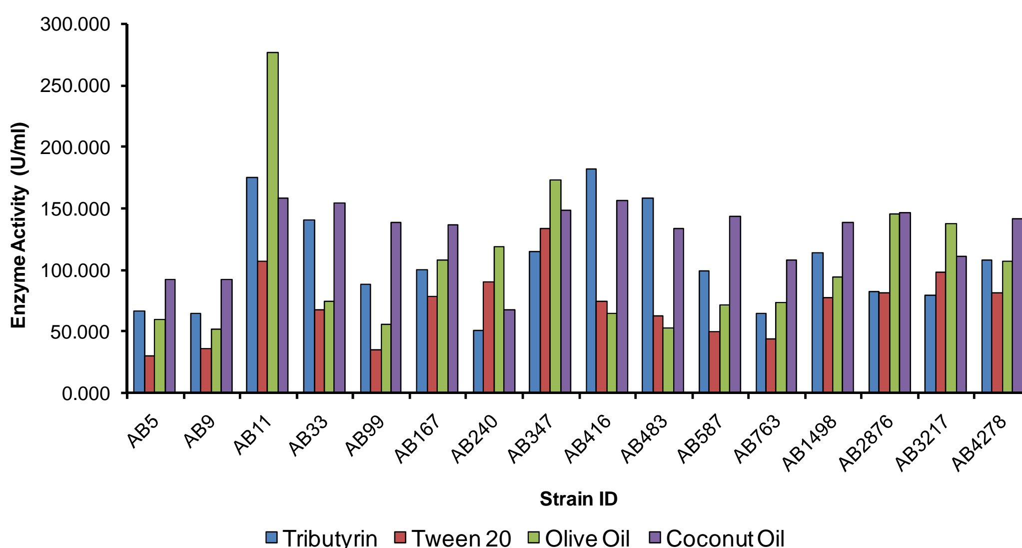


Fig. 6.3. Lipolytic activity testing using four different types of substrates

6.3.2. Phenotypic and molecular identification of the selected strain

6.3.2.1. Morphological and Phenotypic characterization

The cells were gram positive rods with spore formation. The biochemical characteristics of *Bacillus* sp. (AB-11) are presented in table 6.2. The strain was found to be NaCl tolerant, positive for the utilization of glucose, arabinose, xylose,

mannitol, galactose, fructose and mannose. The strain was oxidase and catalase positive and it was capable of producing acid from glucose. The strain showed positive reaction to Voges-Proskauer and Citrate-utilisation tests. The strain was negative for nitrate reduction. Also, it was negative for chitinase, gelatinase and DNAase.

Table 6.2. Biochemical characteristics of *Bacillus* sp. (AB-11)

Bacillus sp.			
Biochemical characterization			
Gram stain	+	Growth at 50°C	+
Motility	+	Growth at 60°C	+
Casein hydrolysis	+	NaCl tolerance (7%)	+
Starch hydrolysis	-	Acid from Glucose	+
Phosphatase	-	Acid+Gas from Glucose	-
Lipase	+	Nitrate reduction	-
DNAse	-	D-Glucose	+
Gelatinase	-	L-Arabinose	+
Chitinase	-	D-Xylose	+
Oxidase	+	D-Mannitol	+
Catalase production	+	Galactose	+
Lecithovitellin reaction	-	Fructose	+
Indole Production	-	Mannose	+
V-P reaction	+	Nitrate	-
Citrate utilization	+	Inositol	-
Anaerobic growth	-	Urea	-

6.3.2.2. Molecular characterization

On the basis of phylogenetic analysis of 16SrRNA gene (1332 base pairs), the strain was identified as *Bacillus pumilus*. The partial 16S rRNA sequences derived in this study have been deposited in GenBank under the accession number KC172396.

6.3.3. Optimisation of Conditions for the enzyme production

6.3.3.1. Effect of Substrate on lipase production

The nature of substrate had significant effect on enzyme production ($p<0.001$). Of the various substrates tested, Olive oil was found to be the optimum

substrate for lipase production (43.36 U). Tween 80 and Tributyrin was also found to have significant influence on lipase production (Fig.6.4).

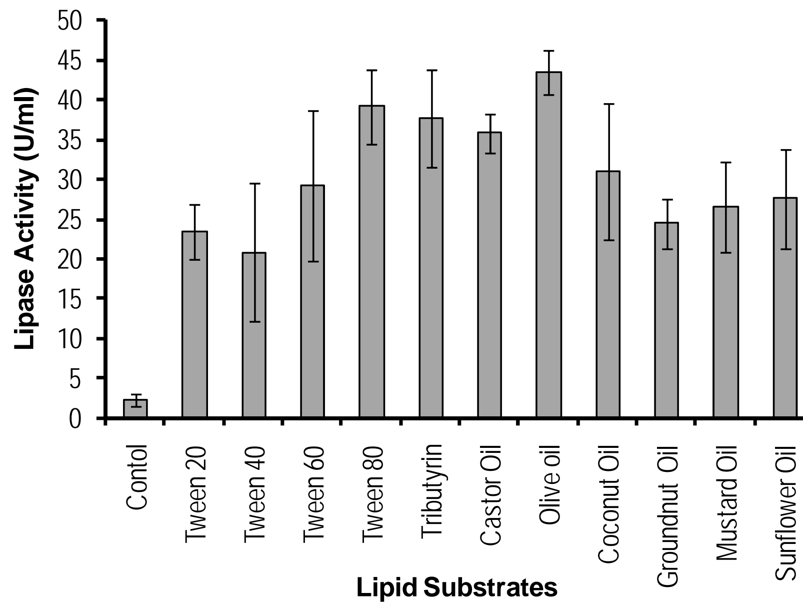


Fig.6.4. Effect of different substrates on the lipase production by Bacillus pumilus

6.3.3.2. Effect of Olive oil concentration on lipase production

The concentration of the olive oil had significant effect on enzyme production ($p<0.001$). Of the various olive oil concentrations tested, the optimum concentration for maximum activity was found to be 2%v/v (Fig.6.5). From the lowest concentration tested, the activity gradually increased and reached the maximum value at 2%v/v and then decreased on further increase of concentration.

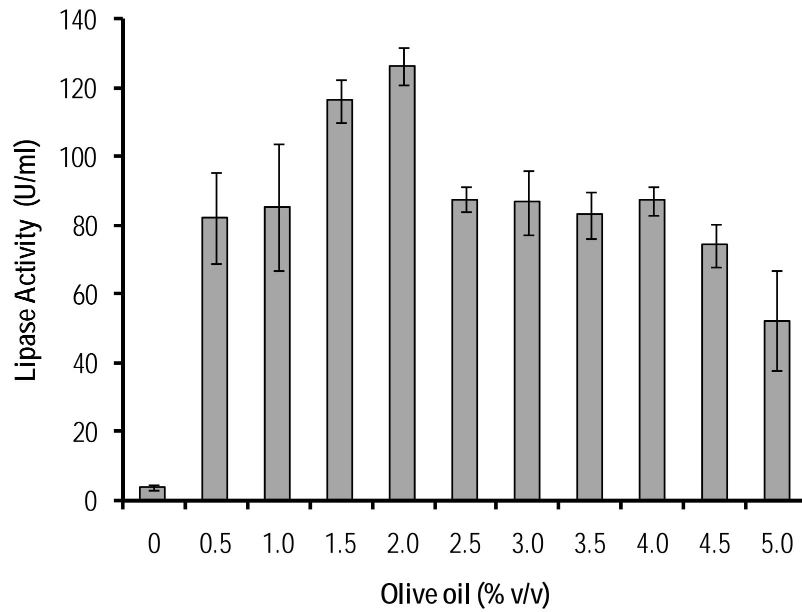


Fig.6.5. Effect of Olive oil concentration on lipase production by B. pumilus

6.3.3.3. Effect of temperature on lipase production

Temperature significantly affected the enzyme production. The optimum temperature for lipase production was found to be 30°C (Fig.6.6). The enzyme production was relatively high in the range of 25 to 60°C.

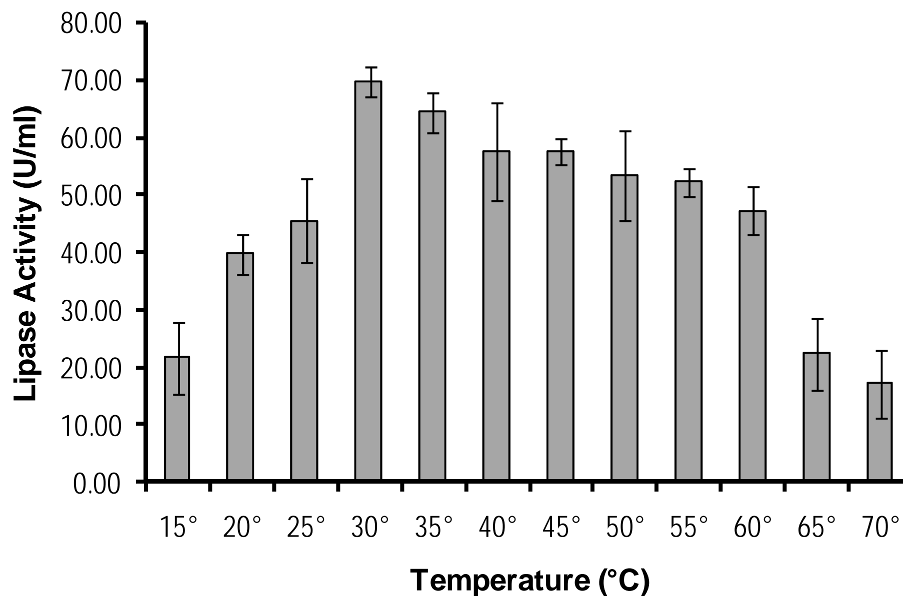


Fig.6.6. Effect of Temperature on lipase production by Bacillus pumilus

6.3.3.4. Effect of pH on lipase production

pH had a significant effect on enzyme production ($p<0.001$). Though the optimum pH for lipase production by *B. pumilus* was 8, there was considerable enzyme production in the pH range 6 to 10 (Fig.6.7).

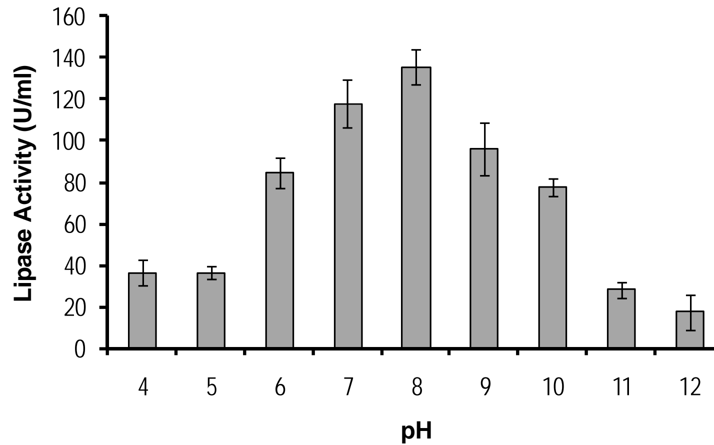


Fig.6.7. Effect of pH on lipase production by Bacillus pumilus

6.3.3.5. Effect of incubation period on lipase production

The time-period of incubation had significant effect on enzyme production ($p<0.001$). The optimum incubation period for maximum lipase production was found to be 24 hours (Fig.6.8). However, the bacterium showed considerable lipase production from 12-48 hours.

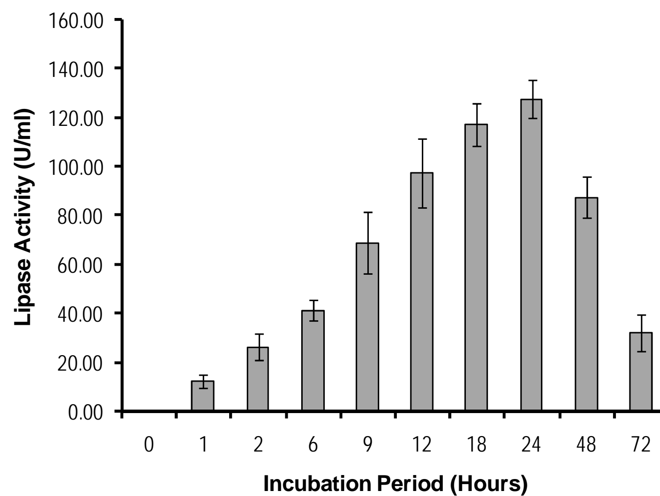


Fig.6.8. Effect of Incubation period on lipase production by B. pumilus

6.3.3.6. Effect of shaking speed on lipase production

The shaking speed of the culture had significant effect on the enzyme production potential of the strain (p<0.001). The maximum enzyme production was obtained at an optimum rpm of 150 (Fig.6.9).

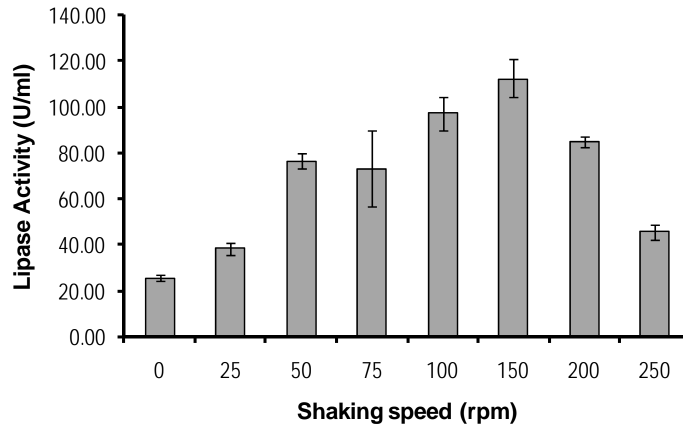


Fig.6.9. Effect of Shaking speed on lipase production by B. pumilus

6.3.3.7. Effect of carbon source on lipase production

The carbon source used for culture had significant effect on enzyme production (p<0.001). Among the various carbon sources used, the best results (96.28 U) were obtained with glucose (Fig.6.10).

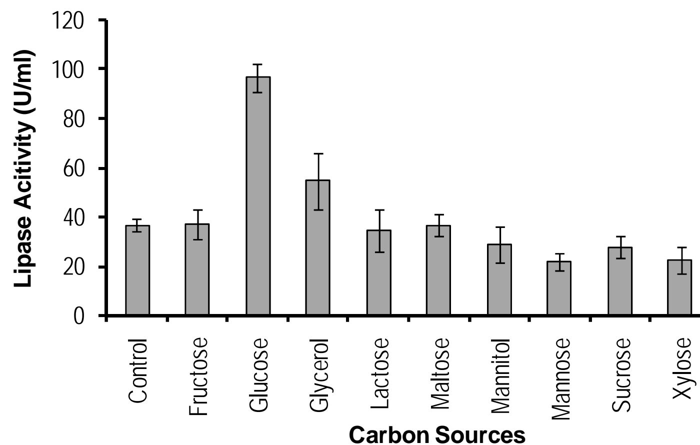


Fig.6.10. Effect of Carbon source on lipase production by B. pumilus

6.3.3.8. Effect of glucose concentration on lipase production

Glucose concentration had significant effect on enzyme production ($p<0.001$). The optimum glucose concentration for maximum enzyme yield was 0.5% (Fig.6.11).

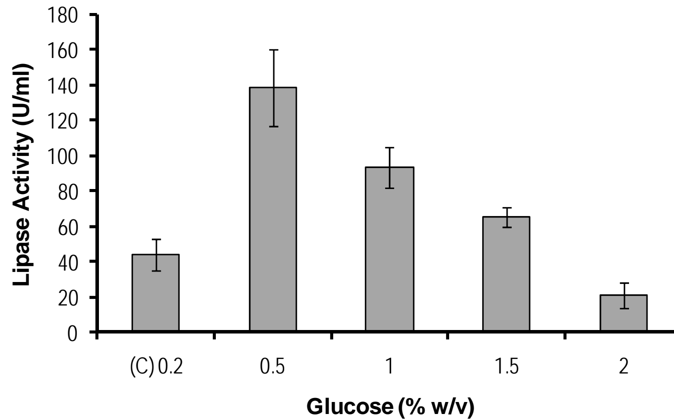


Fig.6.11. Effect of Glucose concentration on lipase production by B. pumilus

6.3.3.9. Effect of nitrogen source on lipase production

Nitrogen source had significant effect on enzyme production ($p<0.001$). Among the various nitrogen sources used, peptone gave the maximum enzyme production (Fig.6.12).

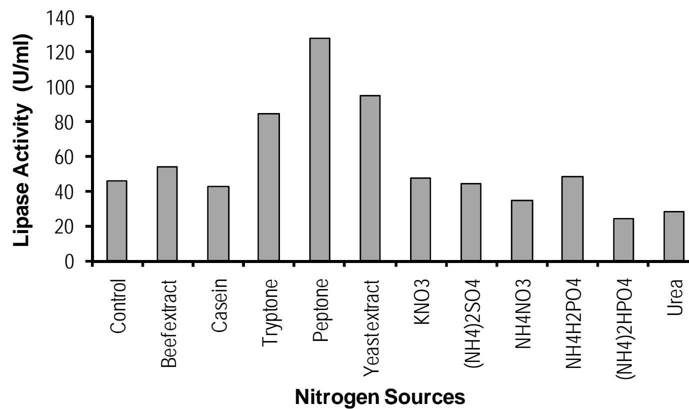


Fig.6.12. Effect of Nitrogen source on lipase production by B. pumilus

6.3.3.10. Effect of peptone concentration on lipase production

Peptone concentration had significant effect on enzyme production ($p<0.001$). Among the various concentrations tested, 0.5% peptone gave the maximum enzyme production (Fig.6.13).

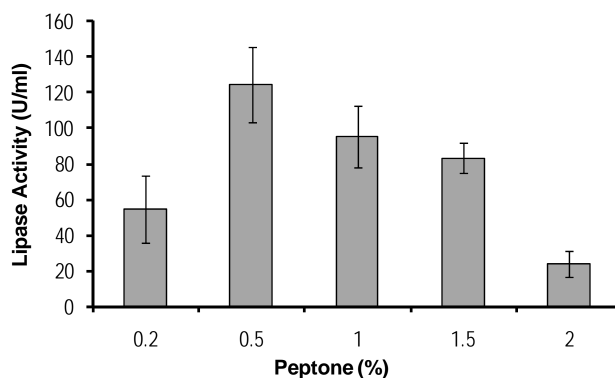


Fig.6.13. Effect of Peptone concentration on lipase production by B. pumilus

6.3.3.11. Effect of NaCl concentration on lipase production

NaCl concentration significantly affected the enzyme production. The optimum NaCl for maximum lipase production was found to be 1% (Fig.6.14). The enzyme production was relatively high in the range of 0.2 to 1%.

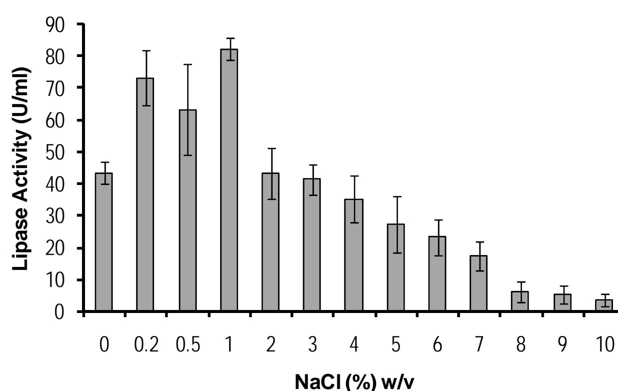


Fig.6.14. Effect of NaCl concentration on lipase production by B. pumilus

Based on the above experiments, the optimum parameters significantly affecting the production of enzyme (Table 6.3) were used for enzyme production, recovery and partial purification.

Table 6.3. Optimum parameters significantly affecting lipase production by B. pumilus

Parameter	Condition	Range
Substrate	**Olive Oil**	**-**
Substrate concentration (% v/v)	**2%**	**0.5-4.5%**
Time (hours)	**24**	**12-48**
Temperature (°C)	**30**	**30-60**
pH	**8**	**6-10**
Shaking speed (r.p.m)	**150**	**50-200**
Carbon Source	**Glucose**	**-**
Carbon Source concentration (% w/v)	**0.5**	**0.5-1.0**
Nitrogen Source	**Peptone**	
Nitrogen Source concentration (% w/v)	**0.5**	**0.5-1.5**
NaCl (% w/v)	**1**	**0.2-1.0**

6.3.4. Partial Purification of enzyme

The lipase isolated from *Bacillus pumilus* AB-11 was purified by ammonium sulphate fractionation followed by gel exclusion chromatography. The lipase could be precipitated with 80% ammonium saturation. Table 6.4 summarizes the data of the purification steps of the extracellular lipase. A purification fold of 3.07 was obtained with ammonium sulphate precipitation and it further increased to 7.42 with gel exclusion chromatography (Sephadex G 100).

Table 6.4. Purification of lipase isolated from *Bacillus pumilus*

Purification Step	Lipase activity (U)	Protein content (mg/ml)	Specific activity (U/mg)	Purification fold
Crude	38.46	0.86	44.72	1.00
$(NH_4)_2SO_4$	87.78	0.64	137.16	3.07
Sephadex G100	145.96	0.44	331.73	7.42

During gel exclusion chromatography on Sephadex G 100, the *B. pumilus* lipase was eluted as an active peak (Fig.6.15). The elution profile revealed major

protein peak between 35 to 45 fractions and the enzyme activity was detected in the fractions 35 to 45. The active fractions were pooled and subjected to further studies.

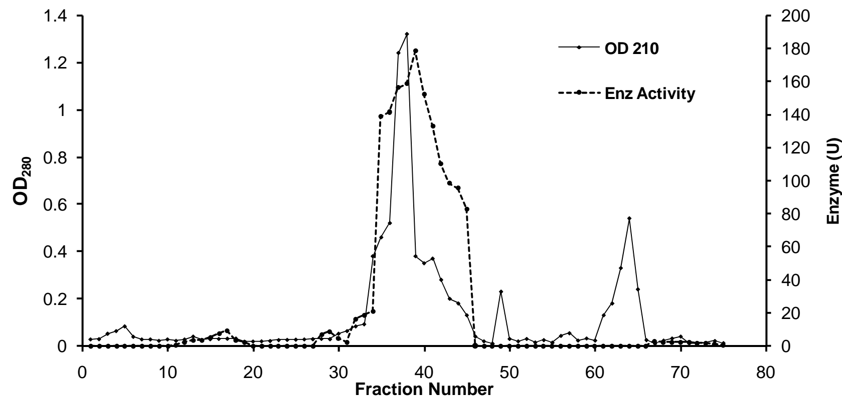
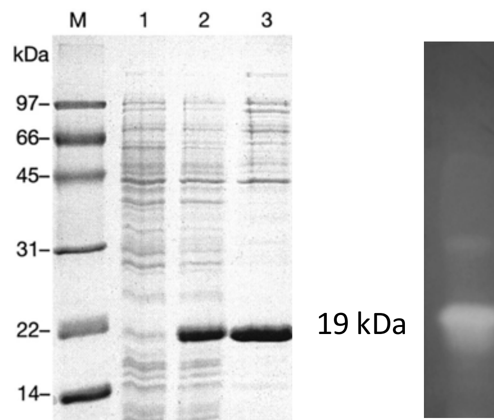


Fig.6.15. Elution profile of B. pumilus lipase from Sephadex G100 column.

The gel exclusion chromatography enhanced the specific activity of enzyme to 331.73 U/mg, compared to 44.72 U/mg of the crude enzyme, thereby attaining 7.42 fold purification. The enzyme could be partially purified and the molecular weight was determined as 19 kDa on SDS PAGE (Fig.6.16).



Coomassie Brilliant Blue Staining Zymogram

M= Mol wt marker
1= Crude;
2= Ammonium sulphate precipitated fraction
3= Sephadex G100 fractions

Fig.6.16. SDS PAGE of B. pumilus lipase and Zymogram of purified lipase

6.3.5. Properties of the purified enzyme

The activity and stability of the purified enzyme under varied pH, temperature, and in presence of surfactants, were analyzed for determination of its possible use in detergent formulation.

6.3.5.1. Effect of pH on the activity and stability of enzyme

The purified enzyme was active in the pH range of 6 to 10, with an optimum at pH 8 (Fig.6.17). The enzyme retained its relative activity till pH 10 (69.55%) indicating the alkaline nature of the isolated lipase. It can be deducted from the data on pH stability (Fig. 6.18) that the lipase exhibited a great deal of stability in the pH range of 6 to 10, with the highest residual activity being observed at pH 8.

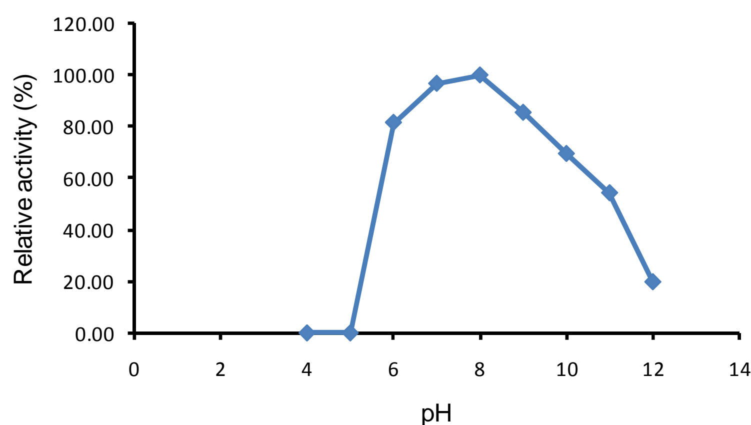


Fig. 6.17. Effect of pH on the activity of lipase from B. pumilus

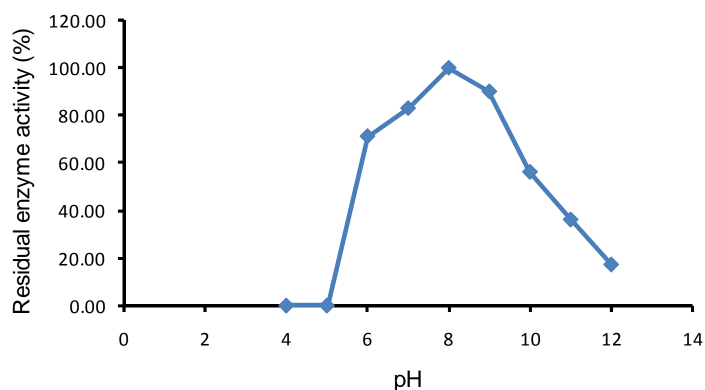


Fig. 6.18. Effect of pH on the stability of lipase from B. pumilus

6.3.5.2. Effect of temperature on the activity and stability of enzyme

The data presented in Fig. 6.19 indicates that the enzyme was active at 30 to 60°C, with the maximum recorded at 35°C. Within the temperature range of 30 to 50°C, the enzyme retained more than 80% of its maximum activity. The enzyme's temperature stability profile (Fig.6.20) revealed a great deal of stability in the temperature range 20 to 60 °C, with the highest residual activity at 35°C.

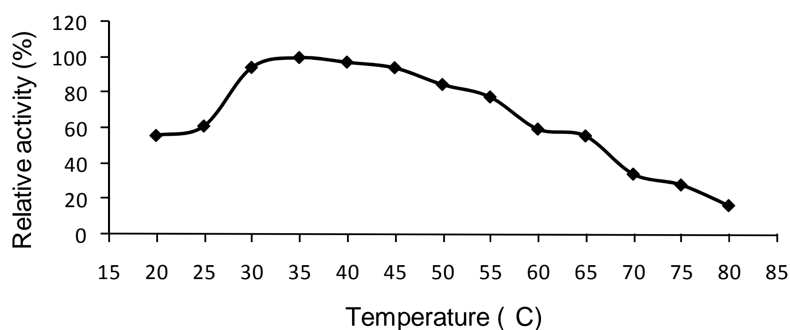


Fig. 6.19. Effect of temperature on the activity of lipase from B. pumilus

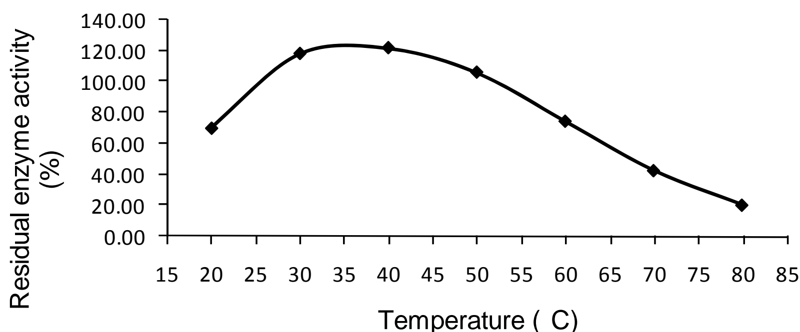


Fig. 6.20. Effect of temperature on the stability of lipase from B. pumilus

6.3.5.3. Effect of surfactants on the activity of enzyme

The stability profile of the enzyme with surfactants (Fig. 6.21) reveals that the enzyme was most stable in Triton X100 at 0.1% level (97% residual activity), closely followed by Tween 20 at 0.1% (96% residual activity). The enzyme showed more than 70% residual activity in presence of all the four surfactants with Triton X100 and Tween 20 having more than 80% residual activity with 1% of the surfactant.

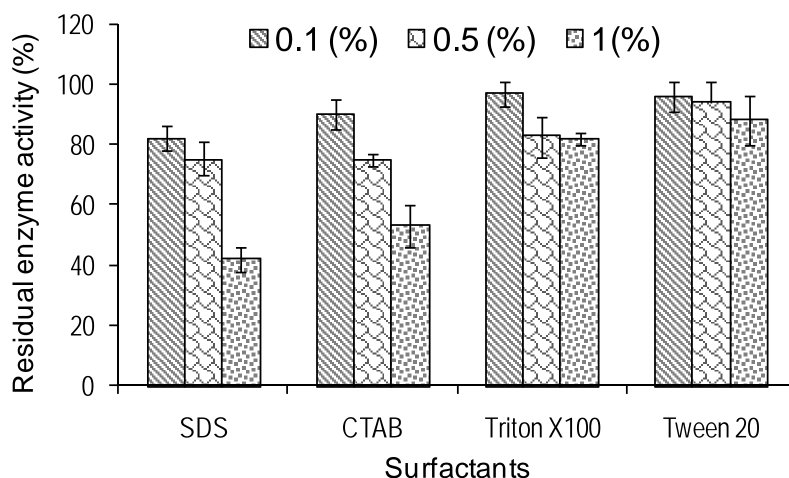


Fig. 6.21. Effect of surfactants on the stability of lipase from B. pumilus

6.4. Discussion

The multiscale development of pharmaceutical, chemical and food industries in the past few decades has resulted in the exploitation of biocatalysts for the synthesis of complex drug intermediates and specialty chemicals. As evidenced from a perusal of available literature, Lipases are the most preferred enzymes today. This acclaim is attributed to their unique properties, including activity over a wide range of temperature and pH, substrate specificity, diverse substrate range and enantioselectivity. Even though the importance of lipases is well acclaimed by industries, such as food, detergents, chemicals, pharmaceuticals, etc., the commercial exploitation of lipases is still in its infancy. This is mainly due to the financial liability involved in lipase purification. Therefore, the present focus should be on development of production and downstream-processing systems which are cost-effective, simple and not time-consuming. Worldwide, research is progressing for isolation for novel lipases. Also, with the advancements in molecular enzymology, substantial efforts are being made for improving the properties of existing lipases for established technical applications and producing new enzymes tailor-made for entirely new areas of application. Considerable progress has been achieved in cloning and biochemical characterisation of novel microbial lipases. Further development is expected with the screening of hitherto unexplored microbial consortia. In this regard, the current study contributes towards the isolation and

purification of a lipase-producing *Bacillus* strain from the coastal region of Kerala, south-west coast of India.

The alkalophilic *Bacillus* strains have often been found to be very good sources of alkaline extracellular enzymes (Ito *et al*., 1998). The lipases from the two mesophilic strains *B. subtilis* and *B. pumilus* were found to be different from other *Bacillus* lipases. They were the smallest true lipases known (approximate molecular mass 20 kDa) and shared very little sequence homology (approximately 15%) with the other lipases (Shah and Bhatt, 2011). The lipase isolated during the current study falls under subfamily 1.4 of Bacillus lipases including low molecular mass lipases in the range 19-20 kDa (Nthangeni, 2001). Lipase purified from a *Bacillus* sp. isolated from oil contaminated soil sample exhibited optimum activity at temperature 60°C and pH 6.0 (Kumar *et al*., 2012). Selva Mohan *et al.*, (2008) indicated that the lipase production varied between *Bacillus* strains and also between varying parameters tested. The maximum lipase production by *B. licheniformis* was reported to be at 50°C after 70 hour of incubation in media containing groundnut meal, casein and neem oil (Prasad and Sethi, 2013). As observed during the current study, among the different nitrogen sources used, peptone was found to be the most suitable nitrogen source for *Bacillus* lipase production (Mukesh *et al*., 2012).

The reasons for the enormous biotechnological potential of microbial lipases are attributed to their ability to act over a wide range of pH and temperature. During the present study, it is found that the lipase isolated from *B. pumilus* was active over a wide range of pH (6 to 10). Moreover, it also exhibited a great deal of stability in the pH range of 6 to 10. Likewise, the enzyme also retained more than 80% of its maximum activity in the temperature range of 30 to 50°C and it revealed a great deal of stability from 20 to 60 °C. These properties indicate the possible use of the isolated enzyme in further application-oriented research.

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. The cleaning power of detergents seems to have peaked. All detergents contain similar ingredients and are based on similar

detergency mechanisms. The lipase of *H. lanuginosa* DSM 3819 is suitable as a detergent additive because of its thermostability, high activity at alkaline pH and stability towards anionic surfactants (Huge and Gormsen, 1987). In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase, which originated from the fungus *T. lanuginosus* and was expressed in *A. oryzae*. Lipases used as detergents also include those from *Candida* (Nishioka *et al., 1990)* and *Chromobacterium* (Minoguchi and Muneyuki, 1989). Laundering is generally carried out in alkaline media, lipases active under such conditions like the *A. oryzae* derived lipase are preferred (Gerhartz, 1990; Satsuki and Watanabe, 1990; Umehara *et al.,* 1990). Alkaline lipase produced by *Acinetobacter radioresistens* had an optimum pH of 10.0 and was stable over a pH range of 6.0 - 10.0, thereby exhibiting great potential for application in the detergent industry (Chen *et al.,* 1998). As part of the present study, the detergent formulation potential of the isolated enzyme was explored with regard to its pH and temperature activity and stability as well as its anionic surfactant stability potential. The enzyme exhibited high activity and stability over a wide range of pH and temperature. Surfactant is one of the major components in detergent formulations (Yangxin *et al.*, 2008). Therefore, the more than 70% residual activity observed with 0.5% each of the surfactants SDS, CTAB, Triton X100 and Tween 20 during the present study is promising. Moreover, the isolated lipase exhibited a stability of 97% with 0.1% of Triton X100. Stability with surfactants would ensure that the full potential of the enzyme is delivered through the detergent formulation. The lipase isolated from *Bacillus pumilus* (KC172396) during the current study is thus active over a wide range of pH and temperature and exhibits stable functioning with surfactant variants. This rapid assessment indicates the potential of the isolated lipase in further research and development activities for development of detergent formulations.

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CHAPTER 7
SUMMARY AND CONCLUSION

The major fraction of the marine environment is hitherto unknown. Marine sediments cover more than two-thirds of the planet thereby constituting the single largest ecosystem on earth in terms of spatial coverage wherein the benthic compartment extends from intertidal region up to the deepest trenches. Microbial cells and prokaryotic activity appear to be the prevalent aspect in these sediments. Microorganisms play major roles in the transformations of metabolic energy and biochemical processes in diverse habitats of marine environments. The benthic substratum is considered to be a location of remineralization and burial of organic carbon. Marine microorganisms have an innate unique metabolic and physiological function that ensures their survival in extreme habitats with a potential for the production of novel enzymes for exploitation of the available nutrient sources of sediments. Out of the enormous bacterial diversity, only a small fraction of marine bacteria have been isolated and cultured. Knowledge of both microbial diversity and microbial activity in the coastal area could be important for understanding the subtle changes occurring in near-shore sediment ecosystems. Coastal zone is considered to be one of the most rich and productive ecosystem. Kerala coast is highly significant for the unique physiographic setting which is responsible for its environmental variability and dynamism. Since there are very few works on distribution of heterotrophic bacteria and their extracellular enzyme production along the coastal ecosystems of Kerala, the present work has focused on the enumeration of the total heterotrophic bacteria, determination of their generic composition and assessment of their enzyme production potential.

During the current study, a total of 2380 bacterial cultures were isolated during 2006-07 and 2412 cultures during 2007-08 from the coastal ecosystems of Kerala. Subsequent identification of the isolated cultures using morphological, physiological and biochemical methods indicated the presence of 24 different genera, including 17 Gram negative and 7 Gram positive forms. Primary screening for

hydrolytic enzyme production indicated that the heterotrophic bacteria isolated during the current study exhibited potential for production of eleven different types of extracellular enzymes, with the maximum number of strains producing protease, followed by amylase and lipase. Among these, 455 strains exhibited potential for lipase-production, of which the 16 most potent strains were taken up for secondary screening. Lipolytic activity testing indicated the strain Ab-11 to have maximum lipase production using the substrates Tributyrin, Tween, Olive oil as well as Coconut oil. On the basis of phylogenetic analysis of 16srRNA gene (1332 base pairs), the strain was identified as *Bacillus pumilus* (KC172396). Optimisation and purification yielded an alkaline lipase of Molecular Weight 19 kDa.Hydrography of near-bottom waters and sediment characteristics are highly significant factors in the biogeochemical cycling in the coastal ecosystems. They in turn determine the diversity and abundance of micro as well as macrobiota. The hydrography as well as sediment characteristics showed significant differences between the stations, as well as between the seasons during the current study. The nutrient parameters did not show any particular trend between the stations. BOD was found to be relatively high in the mangrove stations, as a consequence of the high organic matter (OM) concentration in these stations. The sediment textural analysis indicated that irrespective of seasonal changes, sand, silt and clay dominated the estuarine, coastal and mangrove stations, respectively. The predominance of clay in the mangrove stations could be attributed to the relatively high OM content in these stations. Also, mangrove sediments were found to have high nutritive content (Labile Organic Matter) as compared to coastal and estuarine systems. Further studies on the microbial community and their potential for degradation of organic matter would help to determine the role of microbiota in enhancing the nutritive value of sediment biome as well as biogeochemical cycling of vital elements.

The benthic microbial environment is dominated by a complex widespread network of bacterial populations. The bacteria in the sediment-water interface are of great biogeochemical and ecological importance considering their role in decomposition, mineralisation and subsequent recycling of organic matter. The current study assessed the THB of three distinct ecosystems, coastal, estuarine and mangrove ecosystems, along the Kerala coast. The observed trend in THB density

was coastal > estuary > mangrove. Based on BIOENV results, pH, Nitrate, Lipid, Protein, Biopolymeric carbon and Protein Nitrogen were found to be the key factors driving the THB population during pre monsoon season. During monsoon, salinity was identified as the major decisive factors of THB density. During post monsoon, a set of nine variables, namely, Salinity, pH, Dissolved Oxygen, Nitrite, Nitrate, Phosphate, Silicate, Biopolymeric carbon and Organic Matter, were found to be considerably affecting the THB density. The relative increase in bacterial density in the mangrove stations during enumeration using the Total Direct Count method indicates that the observed low THB in the mangrove stations could possibly be a virtual scenario, resulting from a largely uncultivable bacterial population. Further studies on the generic composition of the sediment bacterial population would help in better understanding of the intricate sediment microbiome and its ecological relevance.

The sediment microbiota is an inevitable component of the marine ecosystem. The understanding of their critical role in basic ecosystem processes such as primary production and remineralization of organic material has led to the prioritization of benthic microbial research. In the present study, a culture-based approach was adapted to determine the bacterial generic diversity in the benthic realm of selected coastal, estuarine and mangrove ecosystems along the Kerala coast. The observed dominance of Gram negative (GN) forms during the present study can be attributed to the samples being collected from the surface layer of near-shore ecosystems, since GN bacteria are known to initially colonize and successfully thrive in dynamic ecosystems with instabilities in physico-chemical properties, while the Gram-positive (GP) forms are found to colonise the relatively undisturbed deeper sediment layers. In the overall percentage dominance of various genera, the GP *Bacillus* was found to be the most dominant genus, followed by the GN *Vibrio*. The relatively high abundance of *Vibrio* in all the stations during monsoon season could possibly be due to increased land runoff and / or sewage discharge. The presence of Enterobacteriaceae in considerable percentage further indicates the possibility of sewage contamination in the near-shore waters. Determination of bacteriological indices enables assessment of the trophic status of the sediment biome and help in assessment of the extent of disturbance taking place in a given region. In the present

study, the Shannon indices ranged from 2.42 to 2.79, indicating rich species diversity. Cluster analysis and MDS plots on a spatial scale indicated the formation of two distinct groups, group one consisting of the mangrove stations, Kadalundi and Puduvaipu, and group two including Dharmadom and all the coastal and estuarine stations. The distinct cluster formation by the two mangrove stations could be attributed to the least generic diversity in these stations probably due to the functional redundancy caused by fast-growing genera, namely *Bacillus, Aerococcus, Enterobacteriaceae, Cytophaga and Pseudomonas.*

Microorganisms are the key regulators of basic ecosystem processes by contributing towards global elemental cycling. Heterotrophic bacteria are considered as the key players in the processes of organic matter recycling, decomposition and mineralization in aquatic environments. The enzyme production potential of the bacteria isolated from the various stations would therefore reflect the trophic status of the given environment. In the present study, the high enzyme production potential of the bacterial strains isolated from the estuarine station, Fort Kochi, during pre monsoon and post monsoon seasons could be attributed to the apparent heterotrophic nature of the Cochin backwater system. High proportions of protease, amylase and lipase producing bacteria were isolated during the study, indicating the rich organic matter composition of the coastal sediments of the study area. Among the three different types of ecosystems, the mangrove ecosystem was found to have the least enzyme producing strains. This could possibly be due to the relatively low number of strains isolated from the mangrove stations. Moreover, the potential enzymatic activity of bacterial strains observed under the laboratory culture conditions may not be identical to the bacterial activity in natural environments. An in-depth analysis of the microbial loop and the inherent enzymatic activity leading to each biogeochemical cycle would be required to assess the health and status of an ecosystem. The present study, therefore, forms a baseline data for further assessment of the coastal ecosystems.

Hydrolytic enzymes are one of the major contributions of marine microbes towards industrial development. Of the various enzymes isolated from marine microbes, microbial lipases are reported to have enormous biotechnological

potential, owing to their ability to act over a wide range of pH and temperature. With the envisaged advancements in detergent industry, major enzyme manufacturers are promoting research on enhanced production of lipase by enzyme engineering, chemical modifications and screening of new microbial strains. Apart from detergent formulations, lipases have significant applications in the designing of novel drugs, oleochemicals etc. The lipase isolated from *Bacillus pumilus* (KC172396) during the current study was active over a wide range of pH (6 to 10). Moreover, it exhibited a great deal of stability in the pH range of 6 to 10. Likewise, the enzyme also retained more than 80% of its maximum activity in the temperature range of 30 to 50°C and it revealed a great deal of stability from 20 to 60°C. These properties along with its more than 70% residual activity with 0.5% each of the surfactants SDS, CTAB, Triton X100 and Tween 20 and 97% stability with 0.1% of Triton X100 indicates the possible use of the isolated enzyme in further application-oriented research towards development of detergent formulations.

The present study, thus, provides a baseline data on microbial diversity of the coastal realms of Kerala, south-west coast of India. The comparative analysis of the three different types of ecosystems, i.e., coastal plains, estuaries and mangroves indicated low bacterial diversity in the mangroves. This could probably be attributed to the constant community shifts experienced by these mangroves as a result of anthropogenic interventions, thereby recommending conservation measures.

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APPENDICES

Appendix I

KEY FOR THE IDENTIFICATION OF HETEROTROPHIC BACTERIA

(Skerman, 1949 and Oliver, 1982)

Appendix II

Media / Reagents / Stains

1. ZOBELL'S MARINE AGAR 2216e

Peptone : 5 gm
Yeast extract : 1 gm
Ferric phosphate : 0.02 gm
Agar : 20.00 gm
50% sea water : 1000 ml
pH : 7.2

2. **NUTRIENT AGAR MEDIUM**

Peptone : 5 gm
Beef extract : 3 gm
Agar : 20 gm
50% sea water : 1000 ml
pH : 7.2

3. MANNITOL MOTILITY AGAR MEDIUM

Mannitol : 26gm .
NaCl : 15gm
Agar : 4 gm
Distilled Water : 1000ml
pH : 7.2

4. MARINE OXIDATION PERMENTATION MEDIUM (MOF)

MOF : 22 gm
Dextrose : 10gm
NaCl : 15gm
Agar : 1.5gm
Distilled Water : 1000ml
pH : 7.2

5. STARCH AGAR MEDIUM

Peptone : 5 gm
Beef extract : 3 gm
Soluble starch : 5 gm
Agar : 20 gm
50% sea water : 1000 ml
pH : 7.2

6. TRIBUTYRIN AGAR MEDIUM

Peptone : 5 gm
Beef extract : 3 gm
Tributyrin : 10 ml
Agar : 20 gm
50% sea water : 1000 ml
pH : 7.2

7. GELATIN AGAR MEDIUM

Peptone : 5 gm
Beef extract : 3 gm
Gelatin : 20 gm
Agar : 20 gm
50% sea water : 1000 ml
pH : 7.2

8. CELLULOSE AGAR MEDIUM

Peptone : 5 gm
Beef extract : 3 gm
CMC : 5 gm
Agar : 20 gm
50% sea water : 1000 ml
pH : 7.2

9. LIGNIN AGAR MEDIUM

Peptone : 5 gm
Beef extract : 3 gm
Agar : 20 gm
50% Sea water : 1000ml
Tannic acid : 5 gm
pH : 7.2

10. PHENOLPHTHALEIN DI PHOSPHATE AGAR MEDIUM

Peptone : 5 gm
Beef extract : 3 gm
Agar : 20 gm
50% Sea water : 1000ml
Phenolphthalein di phosphate : 10 ml of 1% solution
pH : 7

11. ALGINATE AGAR MEDIUM

Peptone : 5 gm
Beef extract : 3 gm
Agar : 20 gm
50% Sea water : 1000ml
Sodium Alginate : 15 gm
pH : 7.2

12. **MINERAL SALT MEDIUM** (composition in g/l)

Na_2HPO_4	-	6 g
KH_2PO_4	-	3 g
NaCl	-	2 g
NH_4Cl	-	1 g
$MgSO_4$.7H2O	-	(1M) 2 ml
CaCl2.2H2O	-	(0.1M) 1 ml
Distilled water	-	1000 ml
pH	-	7.0

13. Gram's Iodine Safranin-'O'

Iodine : 1gm
Potassium iodide : 2gm
Distilled Water: 300ml

14. Safranin-'O'

Safranin-0	:	0.25gm
Distilled water	:	90ml
Ethyl alcohol (95%)	:	10ml

15. Gram's stain

Solution-A

Crystal Violet: 2gm
Ethyl alcohol (95%): 20ml

Solution-B

Ammonium oxalate: 0.8gm
Distilled Water: 80ml
Solution A and B mixed

16. Spore stain

Malachite green: 5gm
Distilled water: 100ml

Appendix IV

NCBI Resources How To

Nucleotide Nucleotide

Advanced

Display Settings: GenBank

Bacillus pumilus strain CU/Sed/Lip/AB-11 16S ribosomal RNA gene, partial sequence

GenBank: KC172396.1

FASTA Graphics

Go to:

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  AUTHORS   Abhilash,K.R. and Saramma,A.V.
  TITLE     Extracellular alkaline lipases from Bacillus pumilus
  JOURNAL   Unpublished
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  AUTHORS   Abhilash,K.R. and Saramma,A.V.
  TITLE     Direct Submission
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Appendix II

Table 2.1A. Physical parameters recorded during various seasons of 2006-07

Stations	Salinity			Temp			pH			DO		
	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M
Kodikkal	35.37	34.33	36.80	26.87	28.77	28.13	8.10	8.20	8.10	5.21	6.33	5.50
Punnapra	33.90	35.27	35.37	28.90	27.43	29.10	8.17	7.85	8.07	5.33	4.42	5.95
Vadi	33.60	34.43	35.33	29.17	25.53	28.07	8.07	8.07	8.17	5.30	4.14	6.02
Mahe	31.70	32.47	36.40	30.20	25.60	28.47	7.93	7.93	8.07	4.99	5.37	5.44
Balathuruth	33.33	10.10	35.30	28.73	29.63	28.53	7.87	8.07	7.93	4.68	6.45	5.31
Azheekode	34.47	10.85	36.27	29.27	28.50	29.47	8.03	7.73	8.07	5.03	6.42	4.78
Fort Kochi	35.30	26.80	36.17	29.83	27.33	28.50	7.87	7.90	8.07	5.00	5.94	5.88
Kavanad	30.40	21.29	35.33	28.50	28.30	27.33	7.87	7.80	8.07	4.87	6.28	5.61
Dharmadom	31.87	5.50	28.13	29.77	27.30	28.97	7.90	8.23	8.03	4.96	3.45	3.93
Kadalundi	33.63	5.87	27.47	28.03	28.47	28.17	7.90	8.17	8.03	5.22	3.47	3.96
Puduvaipu	29.67	4.50	27.13	30.30	27.33	29.67	8.17	8.20	8.10	3.89	3.17	3.61

Table 2.1B. Physical parameters recorded during various seasons of 2007-08

Stations	Salinity			Temp			pH			DO		
	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M
Kodikkal	36.03	32.17	35.20	31.83	26.17	28.77	8.07	7.53	8.20	6.27	5.79	6.04
Punnapra	34.57	32.63	33.30	32.00	27.50	30.13	7.97	7.70	8.13	5.67	5.93	5.51
Vadi	34.47	33.40	35.57	29.23	25.37	28.30	8.17	7.80	8.07	5.31	4.69	5.81
Mahe	35.47	8.07	27.93	32.17	28.60	29.50	7.80	7.70	8.07	5.09	6.29	5.47
Balathuruth	34.50	6.67	27.50	32.20	26.23	27.43	7.77	7.93	8.13	4.56	6.62	3.87
Azheekode	33.73	27.47	34.47	31.33	28.57	28.40	8.13	7.70	8.30	5.11	6.65	3.77
Fort Kochi	35.53	31.77	35.47	30.23	28.40	28.70	8.00	7.83	8.17	4.24	5.61	4.41
Kavanad	28.57	12.90	30.30	31.17	29.30	28.87	8.13	8.13	8.03	5.33	7.42	6.01
Dharmadom	28.57	8.90	28.60	32.30	27.60	29.67	6.70	8.13	8.03	3.37	4.27	3.47
Kadalundi	29.97	7.23	26.57	31.60	27.43	27.60	7.27	7.87	7.97	3.94	3.38	3.47
Puduvaipu	30.37	9.63	28.80	32.23	28.20	30.40	7.60	8.17	7.87	4.25	3.25	3.39

Table 2.2A. Nutrient parameters recorded during various seasons of 2006-07

Stations	NO2 (µmol L^{-1})			NO3 (µmol L^{-1})			PO4 (µmol L^{-1})			SiO4 (µmol L^{-1})		
	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M
Kodikkal	0.12	0.47	1.24	3.40	3.26	1.05	1.70	2.65	1.92	15.98	38.03	3.36
Punnapra	0.26	0.52	2.07	8.73	3.47	2.78	0.27	0.37	0.80	2.60	25.11	1.04
Vadi	0.33	0.38	0.37	13.43	2.19	2.19	0.37	0.39	0.40	8.70	9.92	3.85
Mahe	0.68	0.76	4.52	4.01	4.00	12.65	0.30	5.47	3.69	4.16	33.68	6.11
Balathuruth	0.78	0.46	10.96	11.27	4.73	25.47	0.26	2.02	3.35	29.40	11.34	9.05
Azheekode	0.43	0.40	0.55	4.93	8.51	12.73	0.28	3.49	1.56	15.20	49.05	31.93
Fort Kochi	1.29	0.53	0.75	7.69	4.34	16.20	1.51	3.80	2.59	16.39	18.16	76.37
Kavanad	0.17	0.25	5.90	4.81	1.47	29.40	0.76	0.46	4.99	47.21	132.16	0.39
Dharmadom	0.38	0.35	0.52	4.12	5.83	2.62	1.39	9.50	11.55	4.05	151.65	39.15
Kadalundi	1.38	0.40	0.51	5.06	6.74	1.43	1.48	9.39	16.89	2.92	101.42	61.89
Puduvaipu	1.22	0.35	1.36	3.26	5.18	3.73	1.76	14.89	8.15	3.19	85.34	28.88

Table 2.2B. Nutrient parameters recorded during various seasons of 2007-08

Stations	NO2 (μmol L^{-1})			NO3 (μmol L^{-1})			PO4 (μmol L^{-1})			SiO4 (μmol L^{-1})		
	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M
Kodikkal	0.67	0.35	0.72	3.82	3.55	10.76	0.81	0.38	0.10	5.98	2.03	14.56
Punnapra	0.35	0.43	0.78	2.59	3.91	0.81	0.10	0.68	0.58	7.38	4.52	37.37
Vadi	0.25	0.82	0.32	3.51	4.04	3.78	0.44	0.89	0.59	3.39	6.48	14.39
Mahe	0.28	0.52	0.37	2.58	2.44	2.71	0.68	0.41	0.33	1.11	6.44	73.37
Balathuruth	0.14	0.64	0.36	3.30	3.51	0.81	0.78	0.09	0.20	14.16	6.38	118.50
Azheekode	0.46	0.60	0.48	5.25	2.51	1.28	1.20	0.29	0.05	32.36	2.62	26.92
Fort Kochi	0.79	0.52	0.55	13.47	4.52	6.80	2.88	0.52	0.79	33.79	3.99	14.03
Kavanad	0.27	0.36	0.22	7.02	1.99	5.08	0.54	0.79	0.78	21.72	3.25	29.82
Dharmadom	1.35	0.30	0.65	2.24	4.83	1.45	14.89	4.68	19.08	46.81	25.74	46.85
Kadalundi	0.70	0.45	0.21	1.48	4.79	3.14	8.14	5.31	16.31	18.80	18.50	33.40
Puduvaipu	1.52	0.60	0.62	1.71	5.41	2.46	6.23	8.78	10.83	10.52	11.29	22.25

Table 2.3A. Productivity parameters recorded during various seasons of 2006-07

Stations	Chl (mg/m^3)			PP gC/ m^3/ day			BOD (mg/l)		
	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M
Kodikkal	5.24	8.46	6.33	0.66	3.33	1.30	1.76	1.35	1.77
Punnapra	3.62	7.33	4.38	0.78	1.40	0.60	1.56	0.74	1.65
Vadi	1.50	8.31	13.80	0.28	0.56	0.53	1.16	1.69	1.66
Mahe	8.79	4.80	3.82	0.73	4.39	0.90	1.49	1.42	1.76
Balathuruth	8.21	6.54	12.87	0.98	1.25	0.90	1.07	1.34	1.61
Azheekode	7.70	5.61	3.68	1.15	2.18	1.21	0.96	1.48	1.85
Fort Kochi	5.76	4.72	2.90	1.33	3.25	1.12	1.00	1.77	1.28
Kavanad	5.84	3.91	5.75	0.53	1.26	0.77	0.76	1.25	1.37
Dharmadom	1.27	1.30	1.57	0.44	0.74	0.76	1.83	2.41	1.70
Kadalundi	1.36	1.86	1.83	0.34	0.95	0.68	1.84	2.98	1.51
Puduvaipu	1.38	1.98	2.27	0.41	0.59	0.69	2.61	3.04	1.71

Table 2.3B. Productivity parameters recorded during various seasons of 2007-08

	Chl (mg/m^3)			PP gC/ m^3/ day			BOD (mg/l)		
	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M	Pre-M	Mon	Post-M
Kodikkal	4.76	2.15	3.66	3.20	1.57	0.90	0.47	1.21	1.78
Punnapra	6.61	2.38	3.54	1.89	0.44	1.59	0.86	1.44	1.79
Vadi	2.36	1.85	1.68	0.58	1.70	0.62	0.76	1.26	1.47
Mahe	10.83	1.58	2.65	3.27	0.39	0.21	1.61	1.12	1.92
Balathuruth	6.40	3.33	3.01	2.40	0.53	1.19	1.28	0.84	1.74
Azheekode	3.36	2.95	3.26	0.83	1.82	1.05	1.43	0.77	1.35
Fort Kochi	2.26	2.47	1.43	1.10	1.41	0.90	1.25	0.82	0.93
Kavanad	7.10	2.93	2.37	0.74	0.47	1.00	1.32	0.65	1.01
Dharmadom	1.38	1.73	2.97	0.39	1.04	0.80	2.46	2.51	1.63
Kadalundi	1.66	1.29	2.35	0.64	1.25	0.78	2.27	2.07	1.29
Puduvaipu	1.50	1.58	1.54	0.81	0.89	0.68	2.23	2.08	1.76

Table 2.4A. Sediment temperature and pH recorded during various seasons of 2006-07

Stations	Pre Mon		Mon		Post Mon	
	Sed Temp	Sed pH	Sed Temp	Sed pH	Sed Temp	Sed pH
Kodikkal	26.57	6.65	26.17	6.30	28.47	7.55
Punnapra	28.60	6.88	25.81	6.36	28.87	7.54
Vadi	28.87	6.63	25.44	6.55	29.34	7.51
Mahe	29.90	6.75	25.47	6.63	28.35	7.53
Balathuruth	28.43	6.83	26.17	6.65	28.40	7.59
Azheekode	28.97	6.91	25.91	6.59	28.36	7.55
Fort Kochi	29.53	6.90	26.54	6.69	28.15	7.63
Kavanad	28.13	6.73	24.67	6.30	26.78	7.64
Dharmadom	29.47	6.74	23.71	6.55	28.08	6.65
Kadalundi	27.73	6.66	24.97	6.65	27.58	7.47
Puduvaipu	30.13	6.90	26.87	6.79	29.38	7.70

Table 2.4B. Sediment temperature and pH recorded during various seasons of 2007-08

Stations	Pre Mon		Mon		Post Mon	
	Sed Temp	Sed pH	Sed Temp	Sed pH	Sed Temp	Sed pH
Kodikkal	28.30	6.95	25.31	6.66	28.44	7.78
Punnapra	28.50	6.94	23.22	6.73	28.74	7.72
Vadi	28.47	6.94	25.98	6.94	27.12	7.69
Mahe	29.73	6.87	26.80	7.03	28.24	7.67
Balathuruth	29.83	6.91	26.01	7.06	26.37	7.68
Azheekode	29.80	6.79	25.43	6.98	27.91	7.88
Fort Kochi	29.63	6.83	25.18	7.09	27.97	7.67
Kavanad	29.50	7.65	25.22	6.66	27.57	7.52
Dharmadom	30.27	6.71	26.67	6.94	27.92	8.51
Kadalundi	29.87	6.91	25.37	7.05	26.99	7.63
Puduvaipu	29.57	6.98	26.42	7.20	29.04	7.30

Table 2.5A. Texture of the coastal sediments recorded during various seasons of 2006-07

Stations	Pre-M			Mon			Post Mon		
	Sand	Silt	Clay	Sand	Silt	Clay	Sand	Silt	Clay
Kodikkal	19.73	57.64	22.63	28.09	53.42	18.49	19.73	67.39	12.88
Punnapra	21.26	43.16	35.58	9.00	73.14	17.86	9.55	70.49	19.96
Vadi	3.48	3.25	93.27	70.44	11.70	17.85	30.28	46.20	23.52
Mahe	7.43	13.05	79.52	39.06	46.09	14.85	45.33	32.90	21.76
Balathuruth	14.99	17.78	67.24	92.93	2.55	4.52	57.12	30.14	12.75
Azheekode	28.44	27.95	43.61	16.68	64.61	18.71	17.14	53.10	29.76
Fort Kochi	74.62	14.68	10.70	13.32	52.69	33.98	15.36	49.30	35.34
Kavanad	17.11	6.91	75.98	81.95	7.31	10.74	31.39	46.55	22.06
Dharmadom	9.60	16.88	73.53	8.26	33.92	57.82	6.49	59.12	34.39
Kadalundi	10.49	16.66	72.85	15.59	38.28	46.13	12.42	61.66	25.93
Puduvaipu	4.28	7.34	88.38	17.13	56.30	26.56	13.04	68.50	18.46

Table 2.5B. Texture of the coastal sediments recorded during various seasons of 2007-08

Stations	Pre-M			Mon			Post Mon		
	Sand	Silt	Clay	Sand	Silt	Clay	Sand	Silt	Clay
Kodikkal	46.97	33.17	19.86	26.22	59.77	14.00	20.12	64.11	15.77
Punnapra	8.47	73.27	18.27	9.12	73.75	17.13	40.71	33.41	25.88
Vadi	46.21	39.15	14.64	3.37	81.40	15.23	3.03	77.49	19.48
Mahe	46.10	35.46	18.44	46.04	34.56	19.40	20.71	34.34	44.95
Balathuruth	42.68	36.55	20.77	53.66	35.26	11.08	4.07	73.13	22.80
Azheekode	47.65	28.20	24.15	16.79	62.71	20.49	94.05	3.58	2.36
Fort Kochi	70.02	15.14	14.84	34.75	55.55	9.70	23.28	55.12	21.59
Kavanad	66.77	17.43	15.80	35.25	43.40	21.35	31.27	51.88	16.85
Dharmadom	7.30	56.20	36.50	8.86	31.91	59.23	13.58	41.00	45.42
Kadalundi	13.08	52.22	34.70	14.79	34.23	50.98	20.31	65.66	14.03
Puduvaipu	6.55	17.55	75.90	17.71	42.97	39.32	9.68	65.86	24.46

Table 2.6A. Elemental composition of the coastal sediments recorded during various seasons of 2006-07

Stations	Pre Mon			Mon			Post Mon		
	N%	C%	S%	N%	C%	S%	N%	C%	S%
Kodikkal	0.06	2.45	0.06	0.06	5.79	0.13	0.09	4.38	0.98
Punnapra	0.24	2.21	0.70	0.05	4.21	0.05	0.13	3.70	0.45
Vadi	0.05	4.79	0.13	0.06	1.47	ND	0.20	2.28	1.06
Mahe	0.02	4.06	0.13	0.05	1.45	ND	0.07	2.41	0.08
Balathuruth	0.06	1.64	0.14	0.23	2.63	0.79	0.21	2.29	0.80
Azheekode	0.06	4.74	0.08	0.18	2.32	0.81	0.23	2.59	0.87
Fort Kochi	0.15	4.34	0.17	0.02	2.57	ND	0.18	2.04	0.63
Kavanad	0.13	2.35	0.33	0.15	3.24	0.57	0.05	1.02	0.08
Dharmadom	0.57	1.61	0.29	0.37	4.29	1.55	0.03	0.69	0.16
Kadalundi	0.38	1.12	0.76	0.31	5.15	2.35	0.06	1.46	0.34
Puduvaipu	0.34	2.78	0.18	0.35	4.41	1.83	0.35	5.07	0.19

Table 2.6B. Elemental composition of the coastal sediments recorded during various seasons of 2007-08

Stations	Pre Mon			Mon			Post Mon		
	N%	C%	S%	N%	C%	S%	N%	C%	S%
Kodikkal	0.09	5.05	0.19	0.20	3.05	0.83	0.03	5.78	0.11
Punnapra	0.06	2.13	ND	0.07	3.76	0.16	0.05	4.82	0.18
Vadi	0.20	2.45	1.02	0.23	2.90	0.81	0.04	0.96	0.29
Mahe	0.11	2.63	0.37	0.13	2.14	0.72	0.18	2.17	0.65
Balathuruth	0.11	5.35	0.15	0.13	2.55	0.62	0.52	7.31	0.61
Azheekode	0.19	2.35	0.74	0.14	2.64	0.85	0.27	3.99	1.56
Fort Kochi	0.10	6.93	0.16	ND	3.04	ND	0.12	1.85	0.64
Kavanad	0.21	2.14	0.88	0.03	5.01	0.12	0.10	2.16	1.35
Dharmadom	0.42	4.94	1.78	0.21	3.12	0.96	0.40	5.31	2.20
Kadalundi	0.35	5.92	2.70	0.25	3.59	1.11	0.14	2.44	0.28
Puduvaipu	0.41	5.07	2.11	0.34	4.62	1.91	0.17	2.81	0.33

Table 2.7A. Labile Organic matter (Protein, Carbohydrate and Lipids) of the coastal sediments recorded during various seasons of 2006-07

	Pre-M			Mon			Post Mon		
Stations	LIP	PRO	CHO	LIP	PRO	CHO	LIP	PRO	CHO
Kodikkal	1678.60	1198.59	872.16	2546.62	1199.85	480.47	1506.30	1215.63	814.38
Punnapra	1464.57	1477.57	964.16	3300.48	1426.61	569.15	1798.11	1639.30	747.63
Vadi	1642.07	1210.91	881.94	2315.88	1310.17	294.75	822.49	1315.58	714.30
Mahe	1723.90	1209.15	887.27	1758.38	1333.68	231.31	1241.36	1814.10	574.55
Balathuruth	1429.14	1205.33	679.78	2333.15	1277.22	455.63	1822.97	1747.35	594.81
Azheekode	1450.81	1202.11	1107.00	1768.12	875.41	369.13	737.81	1714.02	681.58
Fort Kochi	1680.30	1199.47	961.12	1002.49	548.58	247.91	884.42	1530.90	537.54
Kavanad	1466.15	1202.40	1061.37	1324.34	596.68	231.14	1231.95	1654.68	534.14
Dharmadom	2678.60	1874.47	1166.82	2317.80	1351.92	755.78	2263.73	4163.55	1568.65
Kadalundi	2464.57	1670.98	1302.06	2231.21	1227.82	863.07	3685.81	5891.89	2088.34
Puduvaipu	2642.07	1810.19	1249.23	2309.96	1268.30	659.18	2361.26	3616.33	1944.00

Table 2.7B. Labile Organic matter (Protein, Carbohydrate and Lipids) of the coastal sediments recorded during various seasons of 2007-08

Stations	Pre-M			Mon			Post Mon		
	LIP	PRO	CHO	LIP	PRO	CHO	LIP	PRO	CHO
Kodikkal	1623.27	958.29	2070.88	2192.38	1650.51	846.73	1659.22	1308.28	717.61
Punnapra	1799.06	1292.92	2441.86	2297.83	1619.03	761.97	3599.39	2379.10	1220.29
Vadi	1607.11	873.09	2092.98	2196.06	1588.16	755.56	1598.18	1045.64	552.54
Mahe	1702.75	860.06	2096.55	2027.41	1591.34	756.58	1706.40	1206.17	600.09
Balathuruth	1135.96	796.92	1885.24	2029.77	1652.19	845.55	1359.55	1286.65	806.09
Azheekode	1635.10	829.19	2309.24	2352.42	1628.57	759.01	3048.84	2012.53	1036.31
Fort Kochi	1725.80	926.03	2160.72	2298.09	1683.86	840.52	2059.99	1353.23	1073.43
Kavanad	1601.50	962.88	2263.90	2185.25	1522.99	701.82	1388.84	905.66	482.94
Dharmadom	2710.28	569.36	2041.42	2214.52	1443.20	622.01	2484.18	3858.67	1958.85
Kadalundi	2394.13	325.18	1973.18	2190.24	1452.29	720.01	3735.18	3137.30	2264.55
Puduvaipu	2689.67	492.23	2059.55	2266.93	1747.19	865.59	3221.60	2571.67	1316.60

Table 2.8A. Organic matter (OM), Biopolymeric carbon (BPC) and Protein Nitrogen (PN) of the coastal sediments recorded during various seasons of 2006-07

Stations	Pre Mon			Mon			Post Mon		
	OM	BPC	PN	OM	BPC	PN	OM	BPC	PN
Kodikkal	1.36	2195.12	191.77	2.55	2690.08	191.98	3.29	2051.14	194.50
Punnapra	1.50	2208.10	236.41	1.90	3402.06	228.26	3.00	1960.89	102.29
Vadi	4.01	2177.67	193.74	2.09	2496.80	209.63	3.94	1547.22	210.49
Mahe	3.72	2240.31	193.46	2.82	2064.81	213.39	2.91	2049.75	290.26
Balathuruth	3.02	1934.38	192.86	1.93	2557.95	204.35	2.31	2461.36	279.58
Azheekode	2.43	2119.94	192.33	1.94	1902.69	140.07	3.35	1665.86	274.24
Fort Kochi	1.98	2232.41	191.91	1.89	1119.83	87.77	3.07	1628.47	244.94
Kavanad	2.48	2113.33	192.38	2.18	1378.09	95.47	3.67	1948.42	264.75
Dharmadom	6.36	2904.17	139.92	5.43	2703.10	216.31	3.84	4365.40	666.17
Kadalundi	6.31	2698.03	107.36	5.36	2620.27	196.45	4.13	6486.72	942.70
Puduvaipu	5.67	2878.24	129.63	5.31	2617.61	202.93	4.07	4320.55	578.61

Table 2.8B. Organic matter (OM), Biopolymeric carbon (BPC) and Protein Nitrogen (PN) of the coastal sediments recorded during various seasons of 2007-08

Stations	Pre Mon			Mon			Post Mon		
	OM	BPC	PN	OM	BPC	PN	OM	BPC	PN
Kodikkal	1.59	2515.37	153.33	2.00	2791.73	264.08	3.82	2172.52	209.32
Punnapra	1.80	2959.57	206.87	2.55	2821.48	259.05	3.06	4353.42	380.66
Vadi	3.08	2470.33	139.69	1.62	2727.47	254.11	2.99	1932.02	167.30
Mahe	2.74	2537.12	137.61	2.88	2602.94	254.61	2.70	2110.86	192.99
Balathuruth	2.36	1996.56	127.51	2.48	2670.12	264.35	3.47	1972.55	205.86
Azheekode	2.57	2556.33	132.67	3.51	2865.92	260.57	2.50	3687.29	322.00
Fort Kochi	2.04	2612.39	148.16	3.27	2884.87	269.42	1.73	2637.45	216.52
Kavanad	2.64	2578.50	154.06	2.35	2665.93	243.68	2.61	1678.58	144.91
Dharmadom	3.65	3128.27	91.10	5.93	2616.87	230.91	4.27	4537.42	617.39
Kadalundi	3.58	2744.21	52.03	5.93	2642.31	232.37	4.36	5244.48	501.97
Puduvaipu	3.10	3082.26	78.76	5.99	2902.56	279.55	3.98	4202.96	411.47

Table 3.1. Total Heterotrophic Bacteria (THB) from the coastal sediments recorded during various seasons of 2006-07 and 2007-08

Stations	THB x 10^5 cfu / g					
	2006-07			2007-08		
	PRM	MON	POM	PRM	MON	POM
Kodikal	66.52	120.67	78.19	33.39	44.65	42.59
Punnapra	86.97	103.91	97.50	35.68	79.33	0.94
Vaadi	92.97	134.99	94.84	45.80	28.23	16.90
Mahe	39.10	78.49	56.45	19.02	150.28	29.67
Balathuruth	19.31	28.08	25.11	13.66	72.87	27.04
Azhikode	33.59	52.76	45.19	25.41	36.18	14.41
Fort Kochi	41.09	56.64	51.31	66.52	108.64	33.89
Kavanad	33.71	57.03	47.47	25.71	44.79	55.73
Dharmadom	6.63	3.63	14.04	8.68	4.84	11.92
Kadalundi	13.34	10.99	3.83	10.34	7.26	7.32
Puduvaippu	6.66	8.42	6.56	7.67	5.17	5.31

Table 4.1A. Generic composition of bacteria during pre monsoon in 2006-07

Stations	No. of Isolates	No. of Genera	*Achromobater*	*Acinetobacter*	*Aerobacter*	*Aerococcus*	*Aeromonas*	*Alcaligens*	*Alteromonas*	*Arthrobacter*	*Bacillus*	*Brevibacterium*	*Chromobacterium*	*Coryneformes*	*Cytophaga*	*Enterobacteriacea*	*Flavobacterium*	*Flexibacter*	*Micrococcus*	*Moraxella*	*Photobactrium*	*Planococcus*	*Pseudomonas*	*Psychrobacter*	*Staphylococcus*	*Vibrio*
Kodikal	90	16	5	3	4	2	4	0	0	6	24	4	0	0	0	5	3	0	2	0	0	1	4	2	2	19
Punnapra	81	14	1	0	2	2	3	2	1	8	21	0	2	0	0	8	0	0	5	0	0	0	9	2	0	15
Vaadi	79	14	2	2	0	0	7	3	0	2	24	0	0	9	2	1	0	2	1	0	0	0	7	0	0	17
Mahe	89	9	4	0	0	0	3	9	0	4	30	0	0	0	0	14	0	0	0	0	0	0	9	4	0	12
Balathuruth	61	11	2	0	1	2	5	0	4	2	19	0	3	0	0	7	0	0	0	0	0	0	2	0	0	14
Azhikode	63	10	0	6	0	1	0	4	0	2	21	0	0	0	0	3	0	0	4	8	0	0	0	3	0	11
Fort Kochi	95	11	0	0	0	0	3	2	0	11	27	5	0	0	0	2	0	0	5	3	0	0	4	2	0	31
Kavanad	55	11	2	0	4	0	0	5	0	7	18	2	0	2	0	0	0	3	0	0	4	0	0	0	2	6
Dharmadom	43	9	5	0	1	0	4	0	0	9	7	6	0	0	0	3	0	0	0	0	0	0	2	0	0	6
Kadalundi	31	7	0	0	0	2	4	0	0	2	8	0	0	0	0	6	0	0	0	0	0	0	2	0	0	7
Puduvaippu	24	4	0	0	0	0	0	0	0	0	9	0	0	0	7	0	0	0	0	0	0	0	6	0	0	2

Table 4.1B. Generic composition of bacteria during monsoon in 2006-07

Stations	No. of Isolates	No. of Genera	*Achromobater*	*Acinetobacter*	*Aerobacter*	*Aerococcus*	*Aeromonas*	*Alcaligens*	*Alteromonas*	*Arthrobacter*	*Bacillus*	*Brevibacterium*	*Chromobacterium*	*Coryneformes*	*Cytophaga*	*Enterobacteriacea*	*Flavobacterium*	*Flexibacter*	*Micrococcus*	*Moraxella*	*Photobactrium*	*Planococcus*	*Pseudomonas*	*Psychrobacter*	*Staphylococcus*	*Vibrio*
Kodikal	76	14	5	3	4	2	4	0	0	6	24	4	0	0	0	5	3	0	2	0	0	1	4	2	2	19
Punnapra	96	16	1	0	2	2	3	2	1	8	21	0	2	0	0	8	0	0	5	0	0	0	9	2	0	15
Vaadi	88	14	2	2	0	0	7	3	0	2	24	0	0	9	2	1	0	2	1	0	0	0	7	0	0	17
Mahe	76	13	4	0	0	0	3	9	0	4	30	0	0	0	0	14	0	0	0	0	0	0	9	4	0	12
Balathuruth	83	9	2	0	1	2	5	0	4	2	19	0	3	0	0	7	0	0	0	0	0	0	2	0	0	14
Azhikode	73	11	0	6	0	1	0	4	0	2	21	0	0	0	0	3	0	0	4	8	0	0	0	3	0	11
Fort Kochi	79	15	0	0	0	0	3	2	0	11	27	5	0	0	0	2	0	0	5	3	0	0	4	2	0	31
Kavanad	63	11	2	0	4	0	0	5	0	7	18	2	0	2	0	0	0	3	0	0	4	0	0	0	2	6
Dharmadom	73	13	5	0	1	0	4	0	0	9	7	6	0	0	0	3	0	0	0	0	0	0	2	0	0	6
Kadalundi	43	8	0	0	0	2	4	0	0	2	8	0	0	0	0	6	0	0	0	0	0	0	2	0	0	7
Puduvaippu	42	6	0	0	0	0	0	0	0	0	9	0	0	0	7	0	0	0	0	0	0	0	6	0	0	2

Table 4.1C. Generic composition of bacteria during post monsoon in 2006-07

Stations	No. of Isolates	No. of Genera	*Achromobater*	*Acinetobacter*	*Aerobacter*	*Aerococcus*	*Aeromonas*	*Alcaligens*	*Alteromonas*	*Arthrobacter*	*Bacillus*	*Brevibacterium*	*Chromobacterium*	*Coryneformes*	*Cytophaga*	*Enterobacteriacea*	*Flavobacterium*	*Flexibacter*	*Micrococcus*	*Moraxella*	*Photobactrium*	*Planococcus*	*Pseudomonas*	*Psychrobacter*	*Staphylococcus*	*Vibrio*
Kodikal	82	14	5	3	4	2	4	0	0	6	24	4	0	0	0	5	3	0	2	0	0	1	4	2	2	19
Punnapra	98	14	1	0	2	2	3	2	1	8	21	0	2	0	0	8	0	0	5	0	0	0	9	2	0	15
Vaadi	80	13	2	2	0	0	7	3	0	2	24	0	0	9	2	1	0	2	1	0	0	0	7	0	0	17
Mahe	78	9	4	0	0	0	3	9	0	4	30	0	0	0	0	14	0	0	0	0	0	0	9	4	0	12
Balathuruth	81	11	2	0	1	2	5	0	4	2	19	0	3	0	0	7	0	0	0	0	0	0	2	0	0	14
Azhikode	90	11	0	6	0	1	0	4	0	2	21	0	0	0	0	3	0	0	4	8	0	0	0	3	0	11
Fort Kochi	108	13	0	0	0	0	3	2	0	11	27	5	0	0	0	2	0	0	5	3	0	0	4	2	0	31
Kavanad	86	10	2	0	4	0	0	5	0	7	18	2	0	2	0	0	0	3	0	0	4	0	0	0	2	6
Dharmadom	71	11	5	0	1	0	4	0	0	9	7	6	0	0	0	3	0	0	0	0	0	0	2	0	0	6
Kadalundi	51	10	0	0	0	2	4	0	0	2	8	0	0	0	0	6	0	0	0	0	0	0	2	0	0	7
Puduvaippu																										

Table 4.2A. Generic composition of bacteria during pre monsoon in 2007-08

Stations	No. of Isolates	No. of Genera	*Achromobater*	*Acinetobacter*	*Aerobacter*	*Aerococcus*	*Aeromonas*	*Alcaligens*	*Alteromonas*	*Arthrobacter*	*Bacillus*	*Brevibacterium*	*Chromobacterium*	*Coryneformes*	*Cytophaga*	*Enterobacteriacea*	*Flavobacterium*	*Flexibacter*	*Micrococcus*	*Moraxella*	*Photobactrium*	*Planococcus*	*Pseudomonas*	*Psychrobacter*	*Staphylococcus*	*Vibrio*
Kodikal	109	16	6	4	5	3	5	0	0	7	27	5	0	0	0	6	4	0	3	0	0	1	5	3	3	22
Punnapra	96	14	1	0	3	3	4	3	1	9	24	0	3	0	0	9	0	0	6	0	0	0	10	3	0	17
Vaadi	93	13	3	3	0	0	8	4	0	3	27	0	0	10	3	1	0	3	1	0	0	0	8	0	0	19
Mahe	103	9	5	0	0	0	4	10	0	5	34	0	0	0	0	16	0	0	0	0	0	0	10	5	0	14
Balathuruth	74	11	3	0	1	3	6	0	5	3	22	0	4	0	0	8	0	0	0	0	0	0	3	0	0	16
Azhikode	74	8	0	7	0	0	0	5	0	3	24	0	0	0	0	4	0	0	5	9	0	0	0	4	0	13
Fort Kochi	98	11	0	0	0	0	4	3	0	13	22	6	0	0	0	3	0	0	6	4	0	0	5	3	0	29
Kavanad	68	11	3	0	5	0	0	6	0	8	21	3	0	3	0	0	0	4	0	0	5	0	0	0	3	7
Dharmadom	52	10	6	0	1	1	5	0	0	10	8	7	0	0	0	4	0	0	0	0	0	0	3	0	0	7
Kadalundi	37	7	0	0	0	3	5	0	0	3	9	0	0	0	0	6	0	0	0	0	0	0	3	0	0	8
Puduvaippu	28	4	0	0	0	0	0	0	0	0	10	0	0	0	8	0	0	0	0	0	0	0	7	0	0	3

Table 4.2B. Generic composition of bacteria during monsoon in 2007-08

Stations	No. of Isolates	No. of Genera	*Achromobater*	*Acinetobacter*	*Aerobacter*	*Aerococcus*	*Aeromonas*	*Alcaligens*	*Alteromonas*	*Arthrobacter*	*Bacillus*	*Brevibacterium*	*Chromobacterium*	*Coryneformes*	*Cytophaga*	*Enterobacteriacea*	*Flavobacterium*	*Flexibacter*	*Micrococcus*	*Moraxella*	*Photobactrium*	*Planococcus*	*Pseudomonas*	*Psychrobacter*	*Staphylococcus*	*Vibrio*
Kodikal	89	16	6	4	5	3	5	0	0	7	7	5	0	0	0	6	4	0	3	0	0	1	5	3	3	22
Punnapra	81	14	1	0	3	3	4	3	1	9	2	0	3	0	0	9	0	0	6	0	0	0	10	3	0	24
Vaadi	74	13	3	3	0	0	8	4	0	3	2	0	0	10	3	1	0	3	1	0	0	0	8	0	0	25
Mahe	92	9	5	0	0	0	4	10	0	5	3	0	0	0	0	16	0	0	0	0	0	0	10	5	0	34
Balathuruth	65	11	3	0	1	3	6	0	5	3	7	0	4	0	0	8	0	0	0	0	0	0	3	0	0	22
Azhikode	69	11	0	7	0	1	0	5	0	3	5	0	0	0	0	4	0	0	5	9	0	2	0	4	0	24
Fort Kochi	78	11	0	0	0	0	4	3	0	6	7	6	0	0	0	3	0	0	6	4	0	0	5	3	0	31
Kavanad	60	10	3	0	0	0	0	6	0	8	4	3	0	3	0	0	0	4	0	0	5	0	0	0	3	21
Dharmadom	66	13	6	0	1	1	5	0	0	10	4	7	3	0	0	4	0	0	2	0	0	0	3	0	3	17
Kadalundi	40	8	0	0	0	3	5	0	0	3	5	0	0	0	0	6	0	0	0	0	0	2	4	0	0	12
Puduvaippu	39	5	0	0	0	2	0	0	0	0	6	0	0	0	8	0	0	0	0	0	0	0	7	0	0	16

Table 4.2C. Generic composition of bacteria during post monsoon in 2007-08

Stations	No. of Isolates	No. of Genera	*Achromobater*	*Acinetobacter*	*Aerobacter*	*Aerococcus*	*Aeromonas*	*Alcaligens*	*Alteromonas*	*Arthrobacter*	*Bacillus*	*Brevibacterium*	*Chromobacterium*	*Coryneformes*	*Cytophaga*	*Enterobacteriacea*	*Flavobacterium*	*Flexibacter*	*Micrococcus*	*Moraxella*	*Photobactrium*	*Planococcus*	*Pseudomonas*	*Psychrobacter*	*Staphylococcus*	*Vibrio*
Kodikal	53	14	4	2	0	2	3	1	0	0	12	0	0	0	2	7	4	0	2	2	0	0	2	3	0	7
Punnapra	64	14	2	0	3	3	4	3	1	4	14	0	3	0	0	4	0	0	7	0	0	0	5	3	0	8
Vaadi	51	13	3	3	0	0	2	4	0	3	13	0	0	6	3	1	0	3	1	0	0	0	2	0	0	7
Mahe	53	9	5	0	0	0	4	12	0	5	9	0	0	0	0	5	0	0	0	0	0	0	4	5	0	4
Balathuruth	51	11	3	0	1	3	7	0	5	3	10	0	4	0	0	3	0	0	0	0	0	0	3	0	0	9
Azhikode	58	11	0	8	0	1	0	5	0	3	11	0	0	0	0	4	0	0	5	4	0	0	1	4	0	12
Fort Kochi	68	13	2	2	0	4	4	3	0	0	13	7	0	0	0	3	0	0	7	4	0	0	5	3	0	11
Kavanad	55	11	3	0	5	0	0	7	0	2	12	3	0	3	0	0	0	4	0	0	5	0	0	0	3	8
Dharmadom	45	11	4	0	1	1	5	0	0	2	9	8	0	0	0	4	0	0	0	0	0	0	3	0	3	5
Kadalundi	27	7	0	0	0	3	5	0	0	3	8	0	0	0	0	3	0	0	0	0	0	0	3	0	0	2
Puduvaippu	32	5	0	0	0	0	0	2	0	0	9	0	0	0	10	0	0	0	0	0	0	0	8	0	0	3

Table 5.1A. Percentage of hydrolytic enzyme producing bacteria during pre monsoon in 2006-07

Stations	Amylase	Cellulose	Chitinase	Ligninase	Lipase	Pectinase	Protease	Xylanase	DNAse	Phosphatase	Alginase
Kodikal	48	8	7	3	35	14	62	6	8	25	3
Punnapra	61	11	6	2	48	2	72	2	1	16	0
Vaadi	45	6	3	3	54	6	66	2	2	7	2
Mahe	54	5	7	2	57	7	68	1	7	21	0
Balathuruth	46	3	2	0	42	4	48	0	2	4	2
Azhikode	52	2	2	1	56	0	44	2	3	7	3
Fort Kochi	67	3	1	0	72	5	78	0	0	11	1
Kavanad	48	2	4	1	24	0	41	2	2	3	0
Dharmadom	37	2	4	3	27	4	36	2	1	5	2
Kadalundi	18	1	3	0	22	3	27	0	0	7	0
Puduvaippu	22	0	2	2	18	5	19	3	2	8	1

Table 5.1B. Percentage of hydrolytic enzyme producing bacteria during monsoon in 2006-07

Stations	Amylase	Cellulose	Chitinase	Ligninase	Lipase	Pectinase	Protease	Xylanase	DNAse	Phosphatase	Alginase
Kodikal	63	11	9	4	46	2	57	8	11	7	2
Punnapra	81	15	8	3	63	3	95	3	1	11	3
Vaadi	59	8	4	4	71	8	87	3	3	9	0
Mahe	71	7	9	3	75	9	64	1	9	8	3
Balathuruth	61	4	3	0	55	5	63	0	3	5	3
Azhikode	69	3	3	1	52	0	58	3	4	9	0
Fort Kochi	68	4	1	0	48	7	55	0	0	9	0
Kavanad	63	3	5	1	32	0	54	3	3	4	2
Dharmadom	49	3	5	4	36	5	48	3	1	7	2
Kadalundi	24	1	4	0	29	4	36	0	0	9	0
Puduvaippu	29	0	3	3	24	7	25	4	3	4	0

Table 5.1C. Percentage of hydrolytic enzyme producing bacteria during post monsoon in 2006-07

Stations	Amylase	Cellulose	Chitinase	Ligninase	Lipase	Pectinase	Protease	Xylanase	DNAse	Phosphatase	Alginase
Kodikal	32	3	4	2	28	2	36	3	2	8	2
Punnapra	42	0	5	0	33	0	45	0	1	12	1
Vaadi	51	0	0	3	46	2	53	2	0	16	0
Mahe	47	4	4	4	52	1	55	2	1	6	0
Balathuruth	62	3	0	2	66	0	64	3	2	5	0
Azhikode	41	0	2	0	72	0	75	5	0	11	3
Fort Kochi	68	5	3	4	57	0	88	3	4	4	0
Kavanad	42	2	0	0	37	1	65	2	0	6	2
Dharmadom	39	0	0	2	28	2	44	2	2	8	0
Kadalundi	31	0	4	4	25	0	31	1	1	3	1
Puduvaippu	27	3	0	2	31	2	28	0	5	5	0

Table 5.2A. Percentage of hydrolytic enzyme producing bacteria during pre monsoon in 2007-08

Stations	Amylase	Cellulose	Chitinase	Ligninase	Lipase	Pectinase	Protease	Xylanase	DNAse	Phosphatase	Alginase
Kodikal	63	3	21	12	53	8	62	2	2	13	2
Punnapra	52	2	11	8	38	4	61	1	0	7	1
Vaadi	42	1	3	4	32	5	53	0	0	9	3
Mahe	79	0	4	7	48	4	82	2	0	5	2
Balathuruth	65	2	6	6	54	7	66	1	3	7	0
Azhikode	58	0	3	9	41	3	62	0	0	6	0
Fort Kochi	81	3	5	13	67	2	77	2	0	3	0
Kavanad	44	0	1	11	33	8	42	0	0	8	2
Dharmadom	37	3	2	6	21	2	33	0	0	11	0
Kadalundi	21	0	3	7	18	3	27	0	0	5	0
Puduvaippu	16	2	6	3	11	4	18	2	0	8	1

Table 5.2B. Percentage of hydrolytic enzyme producing bacteria during monsoon in 2007-08

Stations	Amylase	Cellulose	Chitinase	Ligninase	Lipase	Pectinase	Protease	Xylanase	DNAse	Phosphatase	Alginase
Kodikal	72	0	5	4	47	3	77	7	0	6	2
Punnapra	77	0	4	7	52	7	81	0	0	4	1
Vaadi	64	0	7	11	51	3	67	3	2	8	0
Mahe	83	0	0	4	77	6	78	0	0	12	0
Balathuruth	47	1	5	7	52	2	53	1	0	7	2
Azhikode	60	2	3	0	42	7	67	2	1	5	4
Fort Kochi	47	0	2	0	27	2	55	4	0	6	2
Kavanad	55	0	0	5	41	3	48	3	2	8	0
Dharmadom	42	1	5	7	32	0	36	0	0	5	2
Kadalundi	27	0	0	4	31	2	29	2	0	6	0
Puduvaippu	18	2	2	5	12	4	30	0	0	8	0

able 5.2C. Percentage of hydrolytic enzyme producing bacteria during post monsoon in 2007-08

Stations	Amylase	Cellulose	Chitinase	Ligninase	Lipase	Pectinase	Protease	Xylanase	DNAse	Phosphatase	Alginase
Kodikal	46	3	0	3	39	0	48	2	0	6	3
Punnapra	52	2	2	2	44	3	55	1	3	11	2
Vaadi	43	0	1	2	32	6	41	0	5	3	2
Mahe	38	5	1	0	37	4	42	3	0	5	1
Balathuruth	37	0	2	4	31	2	46	2	6	6	0
Azhikode	42	2	0	7	36	0	52	2	4	6	0
Fort Kochi	47	3	3	0	22	3	62	1	0	7	0
Kavanad	38	0	3	7	31	5	49	4	3	3	2
Dharmadom	33	4	0	3	27	7	38	3	2	5	0
Kadalundi	12	3	1	5	9	0	22	0	2	8	1
Puduvaippu	16	2	1	0	18	4	31	2	0	3	0

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